21, 1996. Applicants enclose a copy of this European Search Report to show the Examiner that both WO 92/11358 and Young *et al*. cited in the search report are also cited on the PTO 1449. EP-A-0 279 582 was cited on the PTO 1449 submitted on December 21, 1992. Therefore, the three publications cited in the European Search Report have been individually cited on PTO 1449s. It is requested that the Examiner cross out reference to the European Search Report for Appln. No. 9204523.5 on the PTO 1449 submitted on June 21, 1996.

Rejection of Claims Under 35 USC §112, First Paragraph

Claims 17, 20, 21 and 24 are rejected under the first paragraph of 35 USC §112, on the basis that the specification does not enable "the production of any protein in the mammary gland for eventual isolation from the cow's milk ... nor are there sufficient teachings in the art for the production of such a transgenic cow." Applicants respectfully traverse the basis for rejection.

The examiner has stated that:

> ... Applicant argues that it is not true that growth to morula/blastocyst stage prior to implantation is necessary. Applicant argues that both skills (direct transfer of microinjected zygotes to the recipient cow and incubation of the microinjected zygote to the morula/blastocyst stage *in vitro* prior to transfer to the recipient cow as disclosed in Krimpenfort *et al*.) were known to the artisan at the time of filing. These arguments are not persuasive.
>
> A declaration may be found non-persuasive if the opinions offered by the expert declarant, such as Dr. Bondioli, are refuted by evidence in the art. In the previous office action, and in this office action, it is maintained that the art does not support declarant Bondioli's opinions. ... Declarant's statements that the issues were economic rather than functions are not persuasive against the backdrop that the only cows argued to produce a heterologous protein in their milk were produced by a method that required the incubation of the microinjected zygote to the morula/blastocyst stage prior to transfer to the mother.

Office action at page 3. Portion in parentheses added.

A. Techniques for producing transgenic cattle were known in the art at the time of filing the present application

First, Applicants note that neither their specification nor claims are limited to producing a transgenic cow by directly transferring the microinjected embryo to a recipient female. Secondly, the claimed invention is enabled by both the specification alone and in combination with techniques that were known in the prior art prior to filing the present application.

As of the September, 1992, the filing date of the present application, methods for the production of transgenic cattle had been reported in the scientific literature and available to persons skilled in the art. It is accepted that the publications directed to producing transgenic cows or sheep did utilize either an *in vivo* or *in vitro* incubation step prior to implanting the embryos into recipient females to carry the fetuses to term. But it must be emphasized that these techniques were known, published and available to skilled persons to use in making transgenic animals, including livestock, such as cattle, sheep, and goats. Therefore, this information, combined with the knowledge of one of ordinary skill in the art, and the present disclosure regarding the constructs containing the 4.2 kb *Sau*3A - *Kpn*1 promoter of the mouse WAP gene, provides an enabling disclosure to produce any protein under the control of this promoter in transgenic cattle.

As a matter of law, the specification need not teach, and preferably omits, what is well known in the art. *See Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). Furthermore, the specification only has to enable a person of skill in the art to practice the claimed invention without undue experimentation. *Id.* Absolute predictability of the outcome is not required. The level of skill in the transgenic art is very high, the methods to practice the invention were known, and considerable experimentation to produce transgenic animals was routine. Applicants recognize, as would others skilled in the art, that

when livestock animals are utilized, the known methods disclosed in these publications could be utilized.

The additional incubation steps after microinjection of the embryos are not so difficult and the results not so unpredictable that a skilled artisan in the transgenic field could not perform these steps without undue experimentation. The additional step of *in vitro* incubation would not require extraordinary skills in embryo manipulations. All that is required is to place the microinjected embryos into a petri dish after microinjection as per the disclosure in any of these publications and incubate them until they reach a predetermined stage of development, which is readily discernible by a skilled person. In regard to *in vivo* incubation of the microinjected embryo, steps of flushing the embryos from the surrogate would be required but these skills would also not require undue experimentation. Failure to specifically delineate these known steps in the present application does not deprive the specification of its enabling character for producing transgenic cattle containing a stably integrated transgene.

For example, each of WO 91/08216 published June 13, 1991; Krimpenfort *et al.*, *Bio/Technology* 9:844-847 (September 1991); Hill *et al.*, *Theriogenology* 37:222 (January 1992); Massey, *J. Reprod. Fert. Suppl.* 41:199 (1990), and Biodioli *et al.*, in *Transgenic Animals* pp 265-273 (1991), all of which are of record in the present application, were published and available to the skilled artisan before the filing date of the present invention. These publications are evidence that prior to the filing date of the present invention, any of the known methods of producing transgenic cattle were available to Applicants to produce transgenic mice, rats, rabbits, pigs, sheep, goats and cows capable of expressing any known protein under the control of the claimed mouse WAP promoter.

Applicants wish to direct the Examiner to the specification on page 15, lines 10-14, where the preferred method of introducing DNA into embryos is microinjection. It is also

stated that these microinjected embryos are then "...allowed to develop into mature transgenic animals." There is no limitation that the microinjected embryos be handled in a particular manner. It is acknowledged that the specific examples disclosing the production of mice and pigs show the direct transfer of microinjected embryos to recipient females; however, the specification **does not limit the handling of microinjected embryos to the direct transfer method.**

Further, Dr. Bondioli's declaration does not characterize the incubation of microinjected embryos as critical to the production of transgenic cattle. But rather he states in paragraph 3 of his declaration that it is his expert opinion that centrifugation of zygotes was critical to producing transgenic cattle as follows:

> In 1987, I participated in a transgenic cattle project as an employee of Granada BioSciences, Inc....The "major breakthrough" which made the production of transgenic cattle possible at that time was the technique of centrifuging zygotes to allow visualization of pronuclei. This method was published by Wall *et al.*, *Biol. Reprod.* 32: 645-651 (1987), and it is described in the Lubon application.

Applicants' examples on pages 28 and 29 of the specification directed to producing a transgenic pig discloses this centrifugation technique.

Dr. Bondioli's review of the specification and his experience as an expert in producing transgenic cattle led him to conclude that:

> [T]he scientific knowledge available as of January of 1991 in combination with the disclosure of the above-captioned application would have enabled a researcher to produce a transgenic cow having a transgene under the control of the "long WAP promoter."

Dr. Bondioli's Rule 132 Declaration at paragraph seven. Applicants submit that the above recited conclusion of Dr. Bondioli took into account what was known prior to the filing of the present invention regarding the incubation of the

microinjected embryos. In fact, Dr. Bondioli states that all of the work from Granada BioSciences, Inc. used the techniques as disclosed in his Exhibits C-E. As the Examiner herself points out, these cited publications used *in vitro* or *in vivo* incubation of microinjected embryos prior to transfer to recipient females. The Examiner is mistaken to read Applicants' invention as excluding the use of these prior art methods. Thus, when Applicants' specification and claims are interpreted properly, it is clear that the art supports rather than undermines the enablement of the claimed invention.

In fact, the integration and expression of transgenes in cattle had been demonstrated and reported in the scientific literature before the date of Applicants' invention. Therefore, a person skilled in the art could make a DNA construct containing the disclosed mouse WAP promoter operably linked to a known DNA sequence encoding a polypeptide for microinjection into a cow embryo using published methods without undue experimentation.

B. <u>The specification enables producing a polypeptide under the control of the claimed WAP promoter in cows</u>

The Examiner only rejects claims that are directed to the expression of "a polypeptide" under the control of the claimed mouse WAP promoter in cows. Applicants assume that the Examiner believes that the introduction of DNA encoding any protein other than protein C into cow embryos is not enabled by the present disclosure.

Applicants point out that several genes encoding diverse heterologous proteins were stably integrated into the DNA of cows as of the filing date of the present application, as evidenced by Krimpenfort *et al.* (lactoferrin), Hill *et al.* (human estrogen receptor and insulin-like growth factor-I) and Massey (human estrogen receptor). Further, documents provided in Applicants' previous response of July 14, 1997 show that the Krimpenfort method resulted in transgenic cows sired by "Herman"

the bull that expressed lactoferrin in their milk. Specifically, the exhibits to the July 14, 1997 response provide a paper trail to show that the method disclosed in the Krimpenfort publication of September 1991 results in a dairy cow that expressed lactoferrin in her milk. Time is needed to produce a cow expressing a polypeptide encoded by the transgene. The evidence provided in the exhibits shows that ultimate expression of the polypeptide in milk was achieved by the methods disclosed in September 1991. No other experimentation was required. Only breeding the animals, screening for transgenic calves, and eventually screening for the production of the polypeptide in the milk were required. Also see the attached abstract from the IBC's Third Annual International Symposium on "Producing the Next Generation of Therapeutics Exploiting Transgenic Technologies" (Exhibit 1).

In another example of the expression of the integrated transgene in cows, Bowen *et al.*, *Biol. Reprod.* 50: 664 (1994), (Exhibit 2), reported that several months after birth, a transgenic calf developed dramatic muscular hypertrophy followed by muscle degeneration. The authors concluded that this phenotype was associated with the expression of the transgene.

Obtaining a stable transgene is the important first step in producing founders and then a herd of cattle that express the transgene in their milk. But once the transgene is integrated, known methods for identifying the transgenic animals and breeding them to obtain offspring that express the transgene does not require undue experimentation. It only requires time and money to obtain a herd of producers of the protein of interest.

C. <u>Transgenic livestock were produced by the direct method of transferring embryos to recipient females after microinjection</u>

Prior to the filing date of the present invention, production of heterologous polypeptides in milk had been

achieved in goats and sheep, species closely related to cattle, using the direct transfer method of microinjected embryos.

Ebert *et al.* (Exhibit 3) enclosed herewith, shows the expression of human tPA in the milk of goats under the control of the murine WAP promoter. The Examiner is directed to page 836, second column, first complete paragraph, where it is stated that the microinjected embryos are "...either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium ... for 72 hours and subsequently transferred to the uterus of recipient females. See Table 1 where 2 animals out of 22 offspring were transgenic as a result of the direct transfer method. Pages 837 and 838 of this publication show that the founder female produced by the direct embryo transfer method produced human tPA in its milk.

Wright *et al.* (Exhibit 4) disclose the expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. On page 833, second column, under the section "Experimental Protocol", it is recited that transgenic sheep were generated essentially as described in Simons *et al.* (Exhibit 5). Copies of both of these publications are herewith enclosed. Simons incubates microinjected eggs for at least 30 minutes to assess any damage to the eggs prior to transfer to the recipient females. There is no indication that the embryos were incubated to reach the morula/blastocyst stage. Wright does not appear to modify this procedure. Thus, Wright provides evidence of the production of human alpha-1-antitrypsin in the milk of transgenic sheep utilizing the direct transfer method.

In view of these publications, there would be a reasonable expectation that a transgenic protein could be produced in the milk of cattle using the direct transfer method of embryo manipulation.

Applicants believe that they have provided evidence and arguments to support their position that their specification enables the skilled artisan to produce a transgenic cow that expresses a polypeptide under the control of the claimed long

WAP promoter using either of the disclosed methods of handling microinjected embryos. Given Applicants' experience with transgenic mice and pigs, one skilled in the art with the specification and techniques known in the art would be able to carry out the procedures described in the specification to produce a transgenic cow capable of expressing a polypeptide under the control of the claimed long WAP promoter with a high level of confidence of success.

In light of the remarks above, Applicants respectfully request the examiner to withdraw the rejection of the claims under the first paragraph of 35 USC §112.

CONCLUSION

Applicants request reconsideration of the claims on their merits and respectfully solicit early notification of an allowance. If Examiner Crouch should have any questions or believes a telephone discussion would expedite prosecution, she is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

March 24, 1998
Date

Jayme A. Huleatt
Reg. No. 34,485

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INTERNATIONAL BUSINESS COMMUNICATIONS

International Business Communications

Exhibit 1

IBC's Third Annual International Symposium on

PRODUCING THE NEXT GENERATION OF

THERAPEUTICS

EXPLOITING TRANSGENIC TECHNOLOGIES

February 5-6, 1997, PGA National Resort & Spa, West Palm Beach, FL

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ibc Infoline

Name: _____

Company: _____

225 Turnpike Road, Southborough, MA 01772-1749, USA Tel: (508) 481-6400

No[redacted]ata on the Production of Recombinant Human Lactoferrin in Transgenic Cattle - From Technical Challenge to Commercial Reality.

Summary of Presentation by Jan H. Nuijens, MD, PhD, Pharming BV, Niels Bohrweg 11-13, 2333 CA Leiden, The Netherlands.

Human lactoferrin (hLF) is an highly cationic, iron-binding glycoprotein (Mr 77,000) of the transferrin family. Human LF was first isolated from milk, but has also been found in many other external secretions such as tears and saliva, as well as in the specific granules of neutrophils. Extensive *in vitro* and some *in vivo* evidence indicates that hLF participates in host defense against infection, iron metabolism and modulation of inflammatory and immune responses, most notably at mucosal surfaces such as those of the gastroinrestinal tract. Most anti-infective and anti-inflammatory activities of hLF are mediated through sequestration of iron and/or interaction with microbial cell wall components and specific receptors on intestinal epithelial cells and lymphocytes, through its highly positively charged N-terminus.

We have chosen to produce recombinant hLF in the milk of transgenic cows. Mammary gland specific expression vectors based on regulatory elements from the bovine αS1 casein gene and either hLF cDNA (Krimpenfort et al, 1991, Bio/Technology 9: 844-847) or the genomic hLF sequences were introduced into the bovine germline by pronuclear injection of one-cell stage embryos. The latter genomic hLF constructs were selected after a process of construct optimization and evaluation in transgenic mice, which expressed hLF in a mammary gland-specific and lactation-restricted fashion in milk (Platenburg et al, 1994, Transgenic Res. 3: 99-108). Human LF was found at relatively low levels in the milk of hLF cDNA transgenic cows, but at levels in the g/L range in the milk of the first lactating genomic hLF founder cow. Based on the correlation of transgene copy number and hLF expression level in transgenic mice, we expect that the hLF expression levels from the other founder animals harbouring a higher copy number will even be higher. The expression of hLF did not affect milk quantity or composition, nor was associated with changes in health or welfare of the lactating cows. Comparison of recombinant hLF with hLF from human milk revealed the proteins to be virtually identical by immunological (double antibody assays; hyperimmunization experiments), functional (iron-binding and -release; binding to LPS, heparin and other physiologically relevant ligands) and structural criteria (analytical chromatography; protein sequencing; spectroscopy; utilization of glycosylation sites).

Based on these observations, we anticipate that recombinant hLF will exert similar if not identical antimicrobial and anti-inflammatory actions *in vivo*. We are currently generating herds for the large-scale production of hLF and focussing on the pharmaceutical, preclinical and clinical development of the protein to evaluate its therapeutic and prophylactic potential in several human pathological conditions.

Feb. 1997

BIOLOGY OF REPRODUCTION 50, 664–668 (1994)

(2)

Transgenic Cattle Resulting from Biopsied Embryos: Expression of c-ski in a Transgenic Calf

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ABSTRACT

Producing transgenic cattle by microinjection of DNA into pronuclei has been inefficient and costly, in large part because of the cost of maintaining numerous nontransgenic pregnancies to term. We designed a system for early identification of transgenic embryos in which biopsies of embryos were assayed by polymerase chain reaction for presence of the transgene before embryo transfer. A total of 2555 embryos were microinjected with one of two DNA constructs. Of the 533 embryos biopsied, 112 were judged to be potentially transgenic and were transferred nonsurgically to recipients, resulting in production of 29 putative transgenic fetuses. One fetus and one calf (7% of offspring) were subsequently shown to be definitively transgenic. The calf was transgenic for a chicken c-ski cDNA, and several months after birth developed dramatic muscular hypertrophy followed by muscle degeneration. This phenotype was associated with expression of high levels of mRNA from the transgene.

INTRODUCTION

The success of modern animal agriculture is largely a result of many centuries of selection for desirable genotypes. With the advent of transgenic technology, it became appealing to consider accelerating the pace of such genetic progress, including modifying the genome of animals in ways that probably cannot be done by classical means. Cattle represent a very substantial fraction of world agricultural output and are therefore an attractive target for genetic manipulation. However, progress in producing transgenic cattle is constrained by a number of factors, including long generation interval, low fecundity, and the expense of such work. Nevertheless, a small number of transgenic cattle have been produced in recent years [1–3]. To date, none of these transgenic cattle has been reported to express RNA or protein from the transgene.

A large part of the expense in conducting transgenic research with livestock is in maintaining to term the recipient animals that harbor nontransgenic fetuses. This is a particularly serious problem with cattle because efficiency, in terms of the fraction of pregnancies that are transgenic, has been very low, and the cost of maintenance is high. Considerable savings could be realized if embryos could be reliably screened for transgenic status before transfer. Here we report production of a transgenic calf and a transgenic fetus derived from embryos that were biopsied prior to embryo transfer to determine transgenic status.

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MATERIALS AND METHODS

Embryo Manipulation

Embryos for microinjection were obtained by either surgical recovery from superovulated cows or by in vitro maturation and in vitro fertilization of oocytes aspirated from ovarian follicles of slaughtered cows. In the former case, cows were superovulated according to a protocol similar to that previously described [4], consisting of intramuscular injections at half-day intervals of 6, 6, 4, 4, 2, 2, 2, and 2 mg of FSH (FSH-P; Schering-Plough, Kenilworth, NJ) beginning near midcycle. Prostaglandin $F_{2\alpha}$ was administered to induce luteolysis, and 100 μg of GnRH was administered intramuscularly at the onset of estrus. Animals were artificially inseminated one-half day and one day after the onset of estrus. Donor cows were anesthetized 48–54 h after the onset of estrus and subjected to midline laparotomy, and embryos were recovered by retrograde flushing of the oviducts with Dulbecco's PBS supplemented with 0.3 mM pyruvate and 0.2% BSA. Embryos derived from in vitro fertilization were generated essentially as described [5] and were microinjected 18–26 h after mixing of oocytes and sperm.

Microinjection was conducted similarly to what has previously been described for pig and sheep embryos [6]. One-cell embryos were centrifuged for 3 min at 12 500 × *g* to polarize cytoplasmic lipid droplets, and then microinjected into one pronucleus with a solution of the DNA fragment (2 ng/μl in 10 mM Tris [pH 7.4], 0.2 mM EDTA). Two-cell embryos were treated similarly and injected into one or both nuclei. The majority of embryos were then processed through the following system. After microinjection, embryos were placed in 50-μl drops of oviduct epithelial cell-conditioned medium [8] under paraffin oil and cultured overnight. The next day, cleaved embryos were transferred to the ligated oviducts of estrous rabbits. Embryo

were recovered from rabbits 6–7 days later (equivalent to 9–10 days from the onset of donor estrus) and evaluated for stage of development. Morulae and blastocysts were microsurgically biopsied by techniques similar to those previously described for producing identical twin cattle [9]. Briefly, embryos were placed in a 10-cm plastic Petri dish containing protein-free PBS, resulting in sticking of zonae pellucidae to the plastic surface. With the aid of a micromanipulator, a small fragment of razor blade attached to a glass pipette was used to cut a biopsy from the side of the embryo, avoiding the inner cell mass. Typically, the biopsy was estimated visually to consist of 10–30 of the embryo's trophoblast cells. While the biopsied embryos remained in culture, the biopsies were analyzed for the presence of the transgene by polymerase chain reaction (PCR). Embryos classified as strong or weak by PCR were transferred nonsurgically to the uterus of recipient cows that generally were one day behind the donor cow in estrous cycle synchrony. In a majority of cases, single embryos were transferred, but often one or two poor-quality embryos were transferred with a good embryo to the same recipient. Pregnancy was diagnosed by ultrasonography 30–40 days after transfer. Most of the recipients that became pregnant after transfer of putative transgenic embryos were subjected to allantocentesis 70–90 days after transfer. For this procedure, a flank laparotomy was performed under local anesthesia, and 20–30 ml of allantoic fluid was aspirated through a 20-gauge needle attached to a piece of polyethylene tubing; the first 5–10 ml of fluid was discarded to minimize contamination with maternal cells. Fetal cells were collected from the fluid by centrifugation, counted, and assayed by PCR. Fetuses from other recipients were either recovered surgically between 90 and 130 days of gestation or allowed to go to term.

Transgenes

Embryos were injected with one of two DNA sequences. The first was a construct designed to express the bovine leukemia virus (BLV) envelope protein under control of an immunoglobulin regulatory element (IgSVBLVenv), and was constructed with use of standard techniques by ligating sequences encoding the BLV gp51 gene into an expression vector consisting of the SV40 early promoter and mouse immunoglobulin heavy chain enhancer (Schnieke, unpublished). Expression of gp51 from this construct in cultured cells was found to be very poor after its use for microinjection into bovine embryos was initiated. The second construct, MSVski/Δ29, consisted of a truncated form of chicken c-ski under transcriptional control of a murine sarcoma virus regulatory region [10, 11]; this construct was provided by Dr. Pramod Sutrave and Stephen Hughes (NCI, Frederick, MD). For microinjection, both constructs were released from plasmid sequences, isolated from agarose gels, and purified by adsorption and elution from glass particles.

PCR and DNA Hybridizations

Embryo biopsies (approximately 10–30 cells) and cells collected by allantocentesis (approximately 500 cells) were placed in 0.5-ml polypropylene tubes in less than 5 μl of PBS. PCR was initiated by adding a mixture of buffer and primers and heating the samples to 95°C for 7 min in a thermocycler (Perkin-Elmer-Cetus, Irvine, CA). The samples were then cooled to 80°C and held at that temperature while nucleotide triphosphates and *Taq* polymerase was added. Samples were then subjected to 35 cycles of amplification (94°C/1 min, 55°C/1.5 min, 72°C/2.5 min) with a final 10-min extension at 72°C. Final concentrations of reactants in a 100-μl volume were as follows: single-strength buffer, 1.5 mM MgCl, 200 μM for each nucleotide, 200 nM for each primer, and 2.5 U *Taq* polymerase. Positive (plasmid) and negative (no DNA) control samples were included in each assay. The primers used for detection of MSVski were 5′-AAGGAATTCTCTAGCACGATTGAG (forward) and 5′ CTCAGTAGAAGCTGGAGATTG (reverse). At completion of amplification, 25–30 μl of each product was electrophoresed in 2% agarose containing ethidium bromide, and fluorescent bands of the appropriate size were scored qualitatively as clearly positive or questionable (faint).

Extraction of genomic DNA and Southern blot analyses were conducted according to standard procedures [12]. Ten-microgram samples of DNA were restricted overnight, electrophoresed in 0.8% agarose, and transferred to nylon membranes (Gene Screen Plus, Dupont, Wilmington, DE) by capillary blotting. These samples were hybridized overnight to chicken c-ski cDNA probes labeled with ^{32}P by the random priming method. After a final wash in single-strength saline sodium citrate/0.1% SDS at 55°C, blots were subjected to autoradiography for 1–3 days.

RESULTS

Production and Screening of Transgenic Embryos

A total of 1573 surgically collected and 982 in vitro-fertilized embryos were microinjected with DNA. Of these, 875 embryos were injected with IgSVBLVenv, a construct designed to express the bovine leukemia virus envelope glycoprotein, and 1680 were injected with MSVski, a vector for expression of a truncated form of chicken c-ski. Of 2046 embryos transferred to rabbits, 1535 (75%) were recovered, and 533 of those had developed into blastocysts of quality sufficient to biopsy (approximately 32% and 15% of the transferred embryos derived from oocytes fertilized in vivo and in vitro, respectively). Transfer of 112 PCR-positive or PCR-questionable embryos into 88 recipients (66, 20, and 2 transfers of one, two or three embryos, respectively) resulted in establishment of 27 pregnancies with 29 fetuses. Twelve of these fetuses were derived from embryos injected with IgSVBLVenv and 17 from embryos injected with MSVski DNA. To investigate the possibility of false negative diagnoses, 84

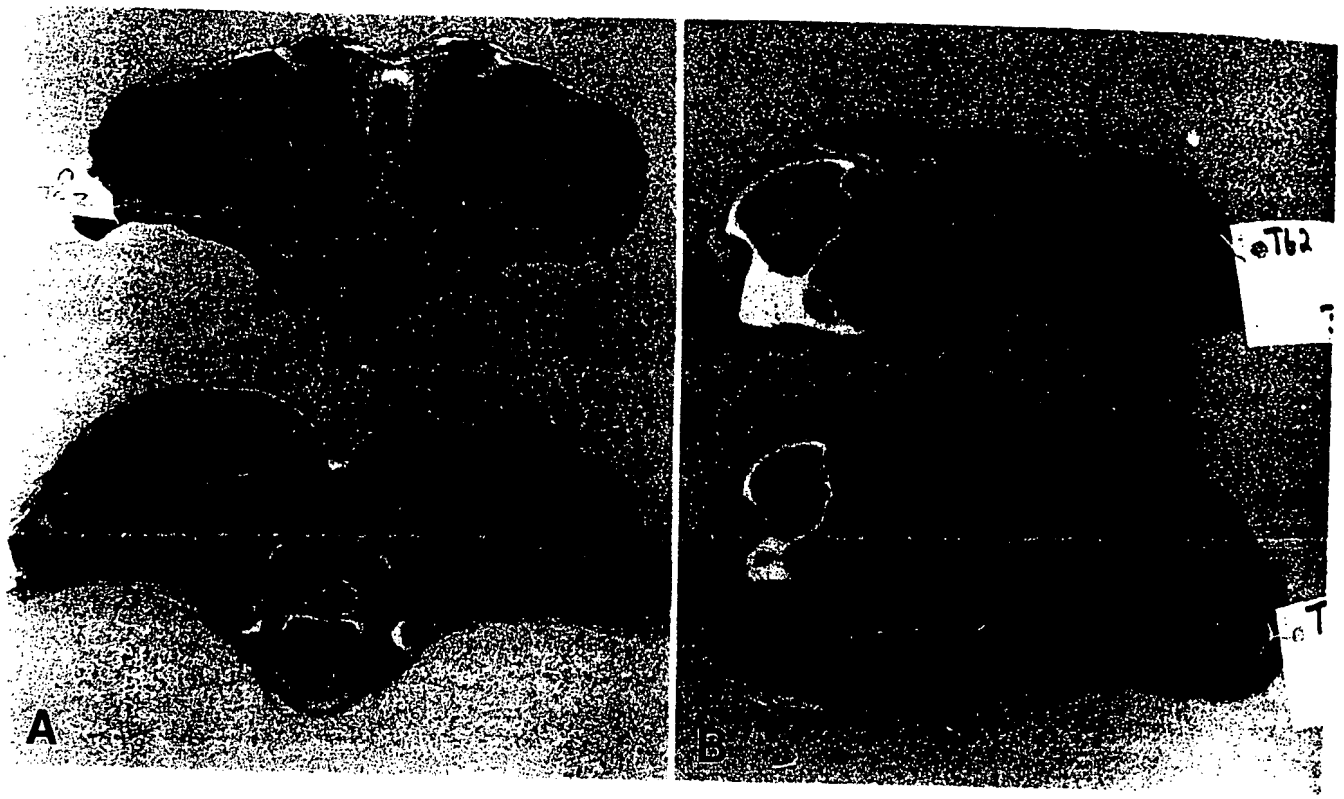

FIG. 1. Characteristics of the carcasses from ski-transgenic calf (bottom of pairs) and his nontransgenic twin sister (top of pairs; sections are at slightly different planes in the carcass). A) Section between 12th and 13th ribs; major muscle is longissimus dorsi. B) Section through shoulder at level of scapulo humeral joint.

biopsied embryos that had been microinjected with MSVski DNA were subjected to PCR analysis after their biopsy was classified as negative. Two of these embryos were classified as putative transgenics on the repeat assay.

Classifying blastocysts as transgenic or not by PCR was of concern because a positive result might only reflect detection of a small amount of injected DNA that had persisted in the embryo but was not integrated. We therefore chose to evaluate each of the putative transgenic pregnancies again at 60–80 days of gestation. Each of the 12 fetuses derived from microinjection of IgSVBLVenv DNA was recovered surgically, and DNA extracted from multiple tissues (liver, skin, and cotyledon) was evaluated by Southern hybridization. One of these fetuses was shown to be transgenic, with the same pattern of hybridizing restriction fragments in all three tissues. The transgene was not detected in the remaining 11 fetuses of this group.

To assess transgenic status in the fetuses from embryos injected with MSVski, fetal cells collected by allantocentesis were again analyzed by PCR. Cells from one of the fetuses contained DNA that was specifically amplified using c-ski primers. This pregnancy consisted of male and female twins. All 17 MSVski pregnancies were allowed to proceed to term. At birth, a skin or tail biopsy was obtained from each of the calves, and DNA extracted from that sample was examined by PCR with c-ski primers. None of the 15 calves diagnosed as nontransgenic at 60–80 days of gestation was transgenic at birth. However, a specific PCR amplification product was obtained with DNA of both twin calves from the pregnancy identified as transgenic by allantocentesis. Southern hybridization with radiolabeled c-ski cDNA and DNA extracted from skin, and at a later age from liver, was used to show that the male calf was transgenic and that the transgene was intact. The female calf showed a small vulva and vagina, and at necropsy, marked uterine and ovarian hypoplasia, characteristics typical of a freemartin. A very weak hybridization signal was obtained with DNA extracted from her skin and a relatively strong signal from liver DNA was undoubtedly due to the hematopoietic chimerism that usually occurs in twin bovine pregnancies; this calf was not considered to be truly transgenic.

Phenotypic Changes Associated with Expression of c-ski

The c-ski transgenic bull calf was normal at birth except for the presence of mild micro-ophthalmia, a defect that is relatively common in cattle and was most likely not associated with his being transgenic. This calf remained phenotypically normal during the first 8 wk of life. However, over the following 2 wk, indications of muscular hypertrophy became evident, particularly affecting the loins and rear quarters. At approximately 10 wk of age, the bull began to manifest sporadic periods of weakness that, over a 2 wk pe-

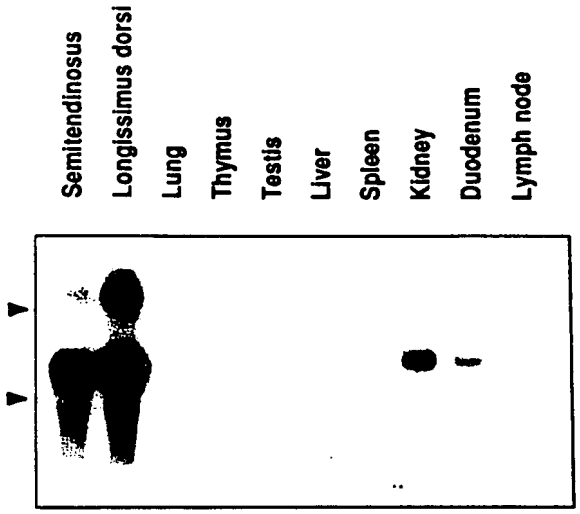

FIG. 2. Northern blot analysis of RNA extracted from tissues collected from ski-transgenic calf. Twenty micrograms of total RNA was loaded in each lane, and blots were hybridized to radiolabeled chicken c-ski cDNA. Arrows mark position of ribosomal RNA bands.

riod, progressed to an inability to stand without assistance. erum concentrations of creatine phosphokinase during this period were significantly elevated (ranging from 808–897 IU/L in comparison to 118–171 IU/L in sera from his nontransgenic twin), suggestive of skeletal muscle degeneration or damage. Throughout this period, the bull remained alert, manifested no signs of pain, and retained a normal appetite. At 15 wk of age, it became obvious that the muscle weakness was not a transient phenomenon, and humane considerations led us to euthanize this animal and, for control purposes, his twin.

Gross evaluation of the carcass from the transgenic bull revealed substantial muscular hypertrophy, which was symmetrical and involved most of the major muscles associated with the axial and appendicular skeleton (Fig. 1). The cross-sectional area of the longissimus dorsi muscle between the 12th and 13th ribs was approximately 40% greater in the transgenic calf than in his nontransgenic twin (50 cm^2 vs. 36 cm^2).

Histopathologic examination of major muscles from the transgenic calf revealed almost all fibers to appear swollen, with a distinctly rounded rather than polygonal outline. A small number of individual fibers were clearly degenerative, with an accompanying mild accumulation of mononuclear cells at their periphery. Many other fibers appeared to be in earlier stages of degeneration, often showing a more acidophilic staining and distinct separation of myofibrils.

Expression of the transgene was assessed by Northern analysis using total RNA (Fig. 2). Messenger RNA for c-ski was present in high concentrations in all axial and appendicular skeletal muscles tested (semimembranosus, semitendinosus, longissimus dorsi, psoas major, triceps, and gluteus medius), as well as in diaphragm. Lesser, but still substantial levels were found in kidney and left ventricle, whereas small quantities were present in small intestine, liver, and spleen. Ski mRNA was not detected in cerebrum, lung, thymus, testis, or lymph node from the ski-transgenic calf, nor in major skeletal muscles (semimembranosus, semitendinosus, and longissimus dorsi) of the nontransgenic twin calf. Transgenic c-ski mRNA was predominantly of the size expected (approximately 2.9 kb), although a larger hybridizing species of unknown origin (approximately 6.5 kb) was observed in muscle samples containing high levels of c-ski mRNA.

DISCUSSION

The study reported here suggests that it is feasible to screen embryos for presence of a transgene prior to transfer into recipients. We generated two transgenic animals from a total of 2555 embryos injected with DNA, an efficiency similar to that reported by other groups using bovine embryos [2, 3]. However, viewed from a different perspective, these two transgenic cattle were derived from 29 fetuses that were obtained from embryos biopsied as blastocysts to assess transgenic status. If the biopsy and PCR step had not been included, we probably would have transferred all 533 good-quality embryos recovered from rabbits to recipient cows (Table 1). Introduction of the embryo biopsy step into the protocol thus did not increase overall efficiency, but it significantly reduced the expense of performing this work by eliminating much of the cost associated with maintaining nontransgenic pregnancies to term.

The PCR technique used for analyzing biopsies is extremely sensitive, but incapable of differentiating integrated DNA from residual input DNA. Short-term persistence in the embryo of small quantities of the microinjected DNA was

TABLE 1. Efficiencies of key steps in producing transgenic calves.

	N	Percent of original embryos	Percent of previous step
Embryos microinjected with DNA	2555	100	100
Embryos transferred to rabbits	2046	80	80
Embryos recovered from rabbits	1535	60	75
Embryos biopsied and subjected to PCR	533	21	35
Embryos transferred to recipient cows	112	4.4	21
Putative transgenic pregnancies	29	1.1	26
Trangenics produced	2	0.1	7

probably responsible for our finding that only 2 of the 29 embryos (7%) that were PCR-positive at biopsy and developed into fetuses were truly transgenic when examined later in gestation. This supposition was supported by PCR on bovine embryos at increasing times after microinjection: 5 of 6, 5 of 6, 5 of 8, 4 of 8, and 2 of 8 embryos were PCR-positive when assayed 1, 2, 3, 5, and 7 days, respectively, after microinjection. Additional experimental support for the problem of plasmid persistence has recently been provided from other laboratories [13, 14]. Despite the problem of false positives, the system of assessing transgenic status in embryos before transfer to recipients reduced the number of recipients needed by 79 percent, as 421 of the 533 good-quality embryos recovered from rabbits were not transferred.

Another important question related to diagnosing transgenic status by embryo biopsy is how frequently the procedure fails to detect truly transgenic embryos, which are then discarded. A substantial percentage of transgenic mice have been found to be mosaics [15]. Integration of a transgene selectively or predominantly into cells that form either inner cell mass or trophoblast could result in a biopsy of trophoblast being falsely classified as negative or positive. We detected the transgene by PCR in 2 embryos from a sample of 84 in which the transgene was not detected in the original biopsy. This could have resulted either from a false negative assay from the biopsy or from transgene mosaicism. Regardless of cause, it is probable that application of the protocol described here will result in discarding an occasional embryo that is truly transgenic. Additionally, there are several ways in which this protocol could be improved, albeit at the cost of increased complexity or time. For instance, a second set of primers could be included as an internal control to co-amplify a segment of bovine genomic DNA. Similarly, Y chromosome-specific primers could be included to sex the embryo, which should allow routine transfer of more than one embryo without the risk of generating freemartins.

The transgenic calf obtained carried a truncated cDNA for the chicken c-ski gene, under regulatory control of murine sarcoma virus promoter and enhancer sequences. This was the same construct used by others to generate transgenic mice [10] and pigs [16]. As might be expected, development of muscular hypertrophy in mice transgenic for chicken c-ski varied with level of expression. The mice that developed muscular hypertrophy showed high levels of c-ski RNA in muscle, whereas those that were transgenic but without the phenotype had low levels of expression. A similar relationship was observed in the c-ski-transgenic pigs. It thus appears that a relatively high threshold of ski gene expression is necessary for development of a hypertrophic phenotype. The ski-transgenic calf reported here supports these observations in that widespread muscle hypertrophy was found in association with high levels of expression of the c-ski transgene. In this calf, as well as in some of the ski-transgenic pigs, high levels of ski gene expression we also associated with muscle dysfunction and degeneratic The pathogenesis of this phenomenon is unclear, partic larly in view of the current lack of understanding of h ski fits into normal patterns of muscle development a interacts with other transcription factors [17–19]. It is like that a window of expression exists in which muscular h pertrophy will develop without impaired function. Belo this window, muscular hypertrophy will not be observe and above it, pathologic changes will ensue. The width this window has significant implications for utility of th gene in transgenic food animals.

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TRANSGENIC PRODUCTION OF A VARIANT OF HUMAN TISSUE-TYPE PLASMINOGEN ACTIVATOR IN GOAT MILK: GENERATION OF TRANSGENIC GOATS AND ANALYSIS OF EXPRESSION

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We report the first successful production of transgenic goats that express a heterologous protein in their milk. The production of a glycosylation variant of human tPA (LAtPA - longer acting tissue plasminogen activator) from an expression vector containing the murine whey acid promoter (WAP) operatively linked to the cDNA of a modified version of human tPA was examined in transgenic dairy goats. Two transgenic goats were identified from 29 animals born. The first animal, a female, was mated and allowed to carry the pregnancy to term. Milk was obtained upon parturition and was shown to contain enzymatically active LAtPA at a concentration of 3 μg/ml.

Several types of human proteins have been expressed in lactating mammary glands of transgenic mice and the proteins secreted into milk (reviewed in refs. 1,2). The scale-up of mouse model systems to livestock species may ultimately provide an alternative production system to the commonly used mammalian tissue culture production processes. A transgenic mammary gland production system would have the potential advantages of not requiring an intensive capital expenditure in setting up a manufacturing facility, and of providing a highly cost efficient system due to high expression levels and low expendable production costs.

Most of the work to date has been done in the mouse model system. Regulatory sequences of the whey acid protein (WAP), β- and α-lactoglobin (BLG) and β-casein genes have been used to target expression of numerous genes to the lactating mammary gland, including tissue plasminogen activator (tPA)[3,4], human anti-hemophilic factor IX[5], soluble CD4[6], human interleukin-2[7], and human α_1-antitrypsin[8]. A vector system based on the αS1-casein gene has also been tested in a mouse model where human urokinase was produced efficiently in milk at levels 10 times greater than those achieved in cellular expression systems[9].

Whereas model systems have demonstrated the feasibility of targeting gene expression to the lactating mammary gland and secretion of heterologous proteins into milk, efficient generation of transgenic livestock and production of foreign proteins in their milk have proven more difficult to achieve. Early work aimed at the generation of transgenic farm animals led to low frequencies of integration, low number of animals that expressed the recombinant proteins, reproductive problems, and resultant physiological problems[2]. By targeting protein synthesis to an exocrine organ in which expressed proteins would be expected to be sequestered away from the circulation and removed from the animal, we and others hope to bypass some of these potential problems in the generation of transgenic livestock. In fact, transgenic sheep, which produce factor IX in their milk, have been generated and apparently exhibit no physiological or reproductive problems[5], although expression levels in these animals were low. More recently, however, high expression of the murine whey acidic protein in transgenic swine and mice may have had adverse effects on the physiology of the mammary gland[10].

We have aimed to produce a commercial prototype for the large-scale manufacture of high market-volume proteins in the transgenic mammary gland system using the dairy goat as a production animal. The goat was chosen for several reasons: (1) Dairy goats produce large volumes of milk, on average 4 liters per day; (2) goats have gestation and development periods of moderate length (5 and 8 months respectively); and (3) goat milk has been extensively characterized at the biochemical level[11]. In this paper, we describe the first successful generation of transgenic goats at frequencies that approach those in the rodent systems. More importantly, a transgenic goat was generated that produced an enzymatically active form of tPA throughout a normal lactation period. These experiments further support the concept of targeting expression of transgenes that encode pharmaceutical proteins to the mammary gland of dairy livestock.

RESULTS AND DISCUSSION

Generation of transgenic goats. The expression vector WAP-tPA was generated previously by fusing a 2·6 kb EcoRI-KpnI fragment upstream of the murine whey acid protein gene to a cDNA encoding wild type human tPA[3]. This vector led to expression of tPA in milk of transgenic mice at levels as high as 250 μg/ml (data not shown). A structural tPA variant was constructed (designated LAtPA) in which an asparagine to glutamine point mutation was introduced into the cDNA to produce a recombinant protein devoid of glycosylation at residue Asn 117. This longer acting tPA variant had an increased systemic half-life in a rabbit model[12]. A DNA fragment containing this point mutation in the tPA cDNA was substituted for the equivalent fragment in WAP-tPA to generate the vector used in this study, WAP-LAtPA (Fig. 1).

Goat embryos were flushed surgically from the oviducts of superovulated dairy goats as described in the Experimental Protocol. The superovulation protocol had been

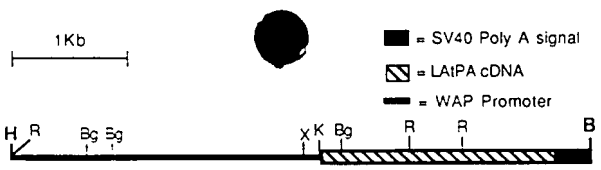

FIGURE 1 A schematic representation of the WAP-LAtPA expression vector and its restriction enzyme sites used for the production of transgenic goats. H = HindIII, R = EcoRI, Bg = BglII, X = XbaI, B = BamHI, K = KpnI.

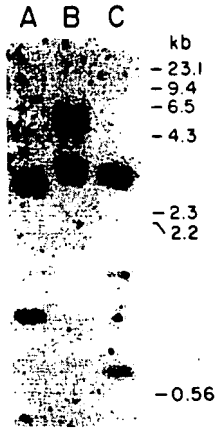

FIGURE 2 Southern blot hybridization of transgenic goat DNA. DNA isolated from the blood of goat #1 was digested, fractionated on an agarose gel, and hybridized to a probe from the whey acid protein gene as described in the Experimental Protocol. Hybridization of this probe to DNA from non-transgenic animals gave no signal (data not shown). Lanes A: BglII; B: XbaI; C: EcoRI.

previously optimized to result in the highest yield and proportion of one-cell embryos[13]. In the course of this study, a total of 372 embryos or ova were collected from 63 donor animals. Of the embryos collected, 252 (68%) were zygotes, 24 (7%) were 2-cells, 5 (1%) were 4-cells and 91 (25%) were unfertilized as determined by the absence of pronuclei. Twenty-eight percent of the injected fertilized embryos were considered poor injections due to the non-optimal positioning of the injected pronuclei, i.e., the pronuclei were at the periphery of the cell making the nuclear injection very difficult. Approximately 8% of the fertilized embryos collected had to be centrifuged for 30 seconds at 13,000 × g to visualize the pronuclei under Normarski optics due to the limited number of recipients and donors available on any given experimental day, microinjected 1-cell, centrifuged 1-cell, and 2-cell goat embryos were typically mixed prior to transfer to the recipient females. This prevented us from confirming the viability of the centrifuged eggs or the potential production of transgenic goats by microinjection of 2-cell embryos. However, subsequent experiments with other fusion genes have confirmed that microinjected centrifuged 1-cell goat embryos are viable and can produce viable offspring. In addition, we have also produced a transgenic goat (potentially a mosaic) that was the result of injection of a 2-cell goat embryo (data not shown).

Embryos were injected with a 4.9 kb HindIII-BamHI fragment of WAP-LAtPA purified free of procaryotic DNA (Fig. 1) at a concentration of 1 μg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA, and either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium containing 10% fetal calf serum in an atmosphere of 5% CO_2 in air at 37°C for 72 hours and subsequently transferred to the uterus of recipient females. Typically the cultured goat embryos were blocked at the 8-16 cell stage in this culture system, but remained viable and could produce live offspring (Table 1).

Pregnancies were confirmed by the inability of recipient animals to return to natural estrus and by ultrasonic examination on days 45 and 55 of pregnancy. Ultrasound on days 45 and 55 of pregnancy have resulted in a 100% early pregnancy confirmation over the first three years of this project. A complete summary of the recipient data is shown in Table 1. Twenty-nine animals were born from 203 embryos transferred, representing 14% of injected embryos surviving to term. Samples of blood and ear tissue from each goat were analyzed by Southern blotting in order to detect transgenics and to confirm that mosaic animals did not escape detection. Two animals were identified as transgenic; a female, goat #1, and a male, goat #21. The generation of 2 transgenic animals in 29 goats born from injected embryos represents an integration rate of 7% and can be compared to frequencies previously reported for mouse (10–30%[14]), rabbit (9.5%[7]), pig (10–40%[15]) and sheep (6–10%[16,5]).

At nine months of age, goat #1 was mated to a non-transgenic male. She became pregnant without difficulty and delivered two non-transgenic progeny. A second pregnancy resulted in three additional offspring, one of which was transgenic. Goat #21 (male) was aspermic and was subsequently diagnosed to have a bilateral spermatocele at the head of the epididymis resulting in blockage of normal, developed sperm from entering the vas deferens;

TABLE 1 Summary of injected goat embryos transferred to recipient females.

A.

No. Recipients	Centrifugation	Transfer To	No. Embryos/Stage	No. Pregnant	No. Offspring	No. Transgenic
28	No	Oviduct	137/1-cell 18/2-cell	13[a]	22	2
2	Yes	Oviduct	10/1-cell	0[a]	0	0
5	No	Uterus	31/morula[b]	4	5	0
1	Yes	Uterus	7/morula[b]	1	2	0

B. Efficiency

No. Recipients	No. Embryos	No. Pregnant (%)	No. Offspring (%)	No. Transgenic (%)
36	203	18 (50.0)[c]	29 (14.3)[d]	2 (6.9)[e]

[a]Two of the pregnant animals aborted prematurely.
[b]Embryos injected at the 1-cell stage and cultured for 72 hours.
[c]Percentage is the number of pregnant animals per number of recipients.
[d]Percentage is the number of offspring born per number of embryos transferred.
[e]Percentage is the number of transgenic animals per live births.

a probable congenital defect that is commonly seen in goats. Therefore, we are unclear as to whether this defect relates to the integration of the transgene.

In order to determine the arrangement of the transgene and to estimate the number of copies per cell, blood DNA from the founder female (#1) was digested with three endonucleases and analyzed by Southern blot (Fig. 2). The blot was probed with a 580 bp EcoRI-BglII fragment from the 5′ end of the WAP upstream region labeled to a specific activity of 1×10^8 cpm/μg of DNA by the random hexamer labeling technique[17]. BglII digestion (Lane A) resulted in a predictable 2.9 kb fragment and a 5′ junction fragment approximately 1.0 kb. BglII is known to cut at three sites within the fusion gene (Fig. 1). The lower intensity of the junction fragment indicates that the integrant was inserted at one site and had multiple copies. XbaI (Lane B) cuts once within the fusion gene resulting in a predictable 4.9 kb fragment if the fusion gene integrated in multiple copies and in a tandem array in normal orientation, as well as 5′ junction fragment of approximately 3.3 kb. EcoRI cuts the fusion gene into three fragments (Fig. 1) of which the 3.3 kb fragment will hybridize to the probe on the Southern blot (Lane C).

Figure 3 shows a Southern blot of an EcoRI digest of DNA extracted from blood cells from goat #1 and her five offspring. The blot was probed with a 1.7 kb LAtPA cDNA fragment. The Southern blot shows 3 major bands representing 3.3 kb, 1.1 kb and 472 bp fragments that corresponds to the 5′, 3′, and interior components of the fusion gene respectively. A minor band of 1.6 kb represents the 3′ junction fragment. The probe is weakly homologous to goat DNA. The initial restriction pattern of genomic DNA from the founder female was consistent with the presence of one to two integrated copies of the tPA transgene. However, as shown in Figure 3, the transgenic offspring was shown to contain more copies of the transgene per cell than the founder. This can be interpreted to mean that the founder animal was a mosaic with more copies of the fusion gene per cell than was originally estimated. Additionally, when the blots were counted on a Beta Scope, the data indicated that the bands in offspring 1-3 were twice the value of the #1 founder and supports the concept that the female founder was a mosaic (data not shown). It is estimated from the Southern analysis that the transgenic offspring contained approximately 3-5 copies of the transgene per cell. It should be noted that Southern analysis of DNA from ear tissue gave identical information.

Expression of tPA in milk. The transgenic mother was milked manually twice per day with an average daily yield of 3-4 liters. Milk was stored frozen at −20°C and thawed just prior to analyses. ELISA and amidolytic assays were run on representative milk samples from the first two months of lactation with continued ELISA assay up to the end of lactation (240 days). The daily volume and concentration of LAtPA is shown in Figure 4A. The animal expressed LAtPA at approximately 3 μg/ml during the peak lactation period (1 to 140 days) with an increase in concentration (6.0 μg/ml) toward the end of the lactation period (141 to 240 days). The second lactation produced LAtPA at the same concentration as the first lactation. The first lactation period was truncated from a normal 300 days to 240 days to eliminate the hand milking procedure. The daily milk output and lactation curve were characteristic of a normal dairy goat during her first lactation. The apparent rise in output of LAtPA during the later part of the lactation period did not parallel the constant total protein concentration in the same milk samples. The elevated milk production from September 17 to November 12 corresponds to the initiation of her second estrus season (first

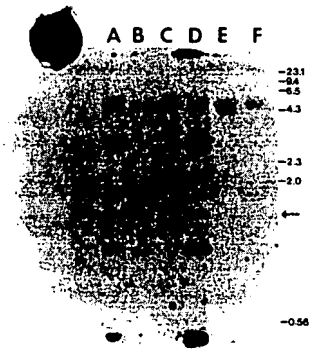

FIGURE 3 Southern blot hybridization of the founder transgenic goat #1 (Lane A) and her five kids (Lanes B, C, D, E, F). DNA isolated from blood was digested with EcoRI, fractionated on an agarose gel, and hybridized to a 1.7 kb LAtPA cDNA fragment. Hybridization of this probe to the transgene shows 3 bands: A: 3.3 kb 5′ junction fragment (Band 1); a 1.1 kb 3′ fragment (Band 2); a 472 bp interior fragment (Band 3). A minor band of 1.6 kb represents the 3′ junction fragment (arrow). Kid #1-3 (Lane D) was shown to be transgenic. Note the intensity of the bands from kid #1-3 was twice as intense as the founder female #1 and indicates that the founder animal is probably a mosaic.

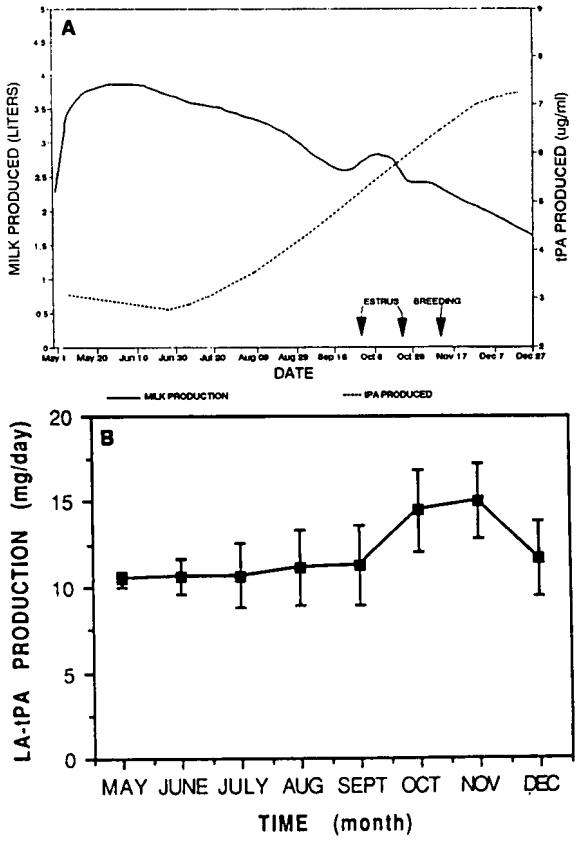

FIGURE 4 (A) LAtPA production throughout lactation in a transgenic goat. The solid line represents the best fit line for daily milk production throughout the 240 day lactation period. The dotted line represents the best fit line for the daily concentration of LAtPA in the milk. The days of her first, second and third estrus cycle and breeding are indicated on the graph. (B) Stability of expression of LAtPA in the milk of a transgenic goat (#1). Milk was harvested and stored as described in the accompanying paper. The concentration of the recombinant enzyme in the milk was determined by ELISA. Total LAtPA produced per day was calculated and daily averages and standard deviations for individual months determined by pooling the data from seven days during the course of each month.

estrus = October 3; second estrus = October 24; third estrus = November 12). The total amount of LAtPA produced per day was calculated and daily averages and standard deviations for individual months are shown in Figure 4B. The output of LAtPA remained relatively constant during the course of lactation, ranging between 11 and 15 mg/day. Although the concentration of the enzyme in milk was relatively low, i.e., approximately 10% of that observed in the recombinant C127 cell line, the continual expression was encouraging since it implied consistent production can be achieved in transgenic animals. The slight rise in output of LAtPA during October and November corresponds to estrus activity. Although the basis for this increase is not known, it is possible that the hormonal changes in the animal associated with the breeding season could have some effect on the expression of the transgene. The female was successfully rebred on November 12 and 13. Interestingly, this period of estrus cyclicity corresponded to a significant rise in the total amount of LAtPA being produced per day. The LAtPA produced in the milk was enzymatically active at approximately 610,000 U/mg. A detailed characterization of the protein is reported in an accompanying paper (Denman et al., *Bio/Technology: This issue*).

These experiments show that targeting transgenes that code for medically important pharmaceutical proteins to the mammary gland of dairy goats is feasible. The level of LAtPA was not high (3 μg/ml) in this first transgenic goat. The likelihood that this goat is mosaic may not allow us to achieve the actual expression level of this gene construct until we generate an F_1 female. However, we have recently produced another female transgenic goat that is producing LAtPA from a β-casein promoter at 2-3 mg/ml (data not shown). At this concentration, the dairy goat may be an economically viable bioreactor for human pharmaceuticals.

EXPERIMENTAL PROTOCOL

Production of transgenic goats. Goats used as donor animals were of either Alpine or Saanen breeds. The timing of estrus was synchronized in the donors with norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS; 6 mg). Prostaglandin was administered after the first 7-9 days to remove endogenous sources of progesterone. At day 13 following progesterone administration, follicle-stimulating hormone (FSH, Schering Corp., Kenilworth, NJ) was given to goats at a dose of 18 mg over three days in twice daily injections (Warren Foote, personal communication). During the anestrus season (after February), the dose of FSH was increased to 24 mg administered similarly over three days in twice daily injections. Twenty-four hours following implant removal, the donor animals were mated several times to fertile males over a two-day period. Recipient animals were synchronized by the same protocols as the donor animals except that a single non-superovulatory injection of pregnant mares serum gonadotropin (PMSG, Sigma, St. Louis, MO) was given on day 13 of progesterone treatment in place of the FSH. From September to January, the recipients received 400 IU PMSG, and from February to April they received 750 IU PMSG. Recipient females were mated to vasectomized males to ensure estrus synchrony. Seventy-two hours following implant removal, embryos were recovered surgically from the oviducts of donors. Embryos were flushed from oviducts associated with ovulated ovaries through a cannula with sterile phosphate-buffered saline and were collected in a petri dish as previously reported[13]. The HindIII-BamHI fragment of WAP-LAtPA was injected into one of the two pronuclei from one-cell embryos or into a nucleus of one blastomere of two-cell embryos at a concentration of 1 μg/ml. Embryos were surgically transferred into the oviducts of the recipient females or to the uteri following a 72 hour culture period.

Identification of transgenic goats. DNA was extracted from the buffy coat recovered from blood of goat #1. Following digestion with restriction enzymes as indicated in the legend to Figure 1, DNA was fractionated and blotted onto nitrocellulose[18]. The probe was a 1.7 kb LAtPA cDNA isolated from the region of the whey acid protein gene 2600 bp upstream of the transcriptional start site[5]. The probe was radioactively labeled by the random primer method[17].

Enzyme activity and protein assays. Plasminogen activator concentrations (amidolytic activity) were determined with an indirect method using the plasmin substrate Val-Leu-Lys-*p*-nitroanilide[12] (S-2251, Helena Labs, Inc.). LAtPA concentration was estimated using the Imubind® tPA ELISA assay kit (American Diagnostics, Chicago, IL) adapted to determine LAtPA in goat's milk.

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THE 1991 BIO/TECHNOLOGY COMPENSATION SURVEY

HOW SIX BIOREACTORS FARED IN T-PA PRODUCTION TRIALS

TRANSGENIC CORNUCOPIA: PLUMS, CARNATIONS, AND MELONS

A NEW BACULOVIRUS INSECTICIDE

Exhibit 1

GENERATION OF TRANSGENIC DAIRY CATTLE USING *'IN VITRO'* EMBRYO PRODUCTION

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We have combined gene transfer, by microinjection, with *'in vitro'* embryo production technology, enabling us to carry out non-surgical transfer, to recipient cows, of microinjected embryos that have been cultured from immature oocytes. Using this approach, we have established 21 pregnancies from which 19 calves were born. Southern blot analysis proved that in two cases the microinjected DNA had been integrated in the host genome.

Heterologous protein production in the mammary gland of dairy animals may become an important alternative to cell-culture based expression systems. The feasibility of this technology in the mouse system has been demonstrated in several reports in which high level, tissue-specific expression of foreign proteins in milk was obtained[1–5]. Several groups have reported limited success of germline transformation by applying essentially the same approach to other species, in particular animals with a relatively short generation time such as rabbits, sheep and pigs[6–11]. In these experiments, the supply of fertilized oocytes was, like that in the murine system, based on superovulation followed by *'in vivo'* fertilization. After surgical removal of the zygotes followed by microinjection, embryos were allowed to develop in the oviduct of recipients into which they were placed via another surgical procedure.

Dairy cows would seem to be the optimal species for production of very large quantities of heterologous protein in the mammary gland since they can produce over 10,000 liters of milk per year that contains 35 grams of protein per liter[12]. However, the generation interval of cattle is about 2 years and cows normally produce only one offspring per gestation. In addition, the logistics of supplying the large numbers of bovine zygotes that are required for the production of transgenic animals from live animals using conventional procedures is cumbersome. Moreover, superovulation and artificial insemination followed by flushing of oviducts of donor cows and oviductal transfer to recipients is very costly because of the two surgery steps involved.

Here we report the generation of transgenic dairy cattle based on a novel approach in which gene transfer is combined with an *'in vitro'* embryo production procedure, thus enabling non-surgical transfer of microinjected embryos that have developed from immature oocytes. Using this approach we demonstrate, for 2 cases, the successful incorporation of microinjected DNA in the genome. The integrated DNA construction contains signals for directing expression of the human iron binding protein, lactoferrin, to the mammary gland.

RESULTS

Oocyte maturation and fertilization. Bovine oocytes were collected by aspiration of follicles present on ovaries obtained from local slaughterhouses. For this study a total of about 2500 oocytes were used. On average we performed two aspiration sessions per week. The yield of aspirated oocytes was highly variable from day to day, with a mean daily number of about 150. Maturation and fertilization were analyzed by cytological analysis. Maturation was defined as the breakdown of the nuclear membrane, the appearance of the first polar body and a metaphase plate. For fertilization, frozen semen from three different bulls was used, each with excellent characteristics with respect to genetic background, field perfor-

TABLE 1 Efficiencies of the steps involved in the process from immature bovine oocytes to transgenic calves.

Step	Total No.	Percent*
oocytes	2470	—
matured	2297	93
fertilized	1358	61
injected	1154	85
survival	981	85
cleavage	687	70
transferred	129‡	19
pregnant	21	21
integration	2	10

*Percentages indicate the proportion of embryos or cells that successfully complete each step.
‡Sixty-nine transfers of single blastocytes resulting in 7 pregnancies; 30 transfers of twinned embryos, resulting in 14 pregnancies.

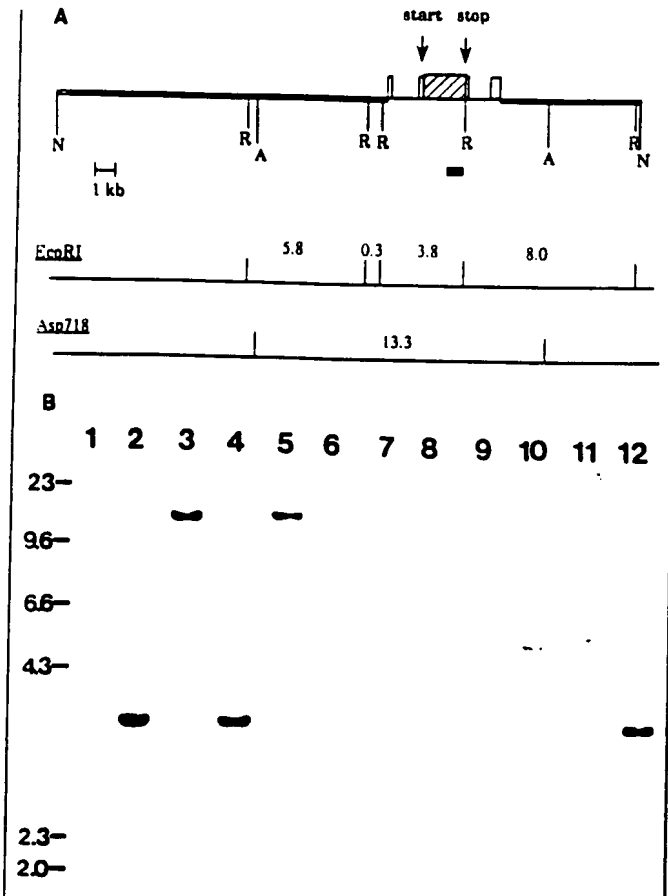

FIGURE 1 (A) Structure of the bovine casein-hLF transgene. The coding sequence of the hLF cDNA is depicted by a hatched box. The position of the translational start and stop codon is indicated. The 5' and 3' untranslated regions are encoded by αS1-casein exons (open boxes). Intervening sequences interrupting these exons are represented by a single line. The expression unit is surrounded by flanking sequences derived from the bovine αS1-casein gene (indicated by a double line). Positions of restriction enzyme sites are indicated by the following symbols: R, EcoRI; A, Asp718; N, NotI; The NotI sites are not present at the indicated positions in the bovine αS1-casein gene itself, but were introduced by synthetic linkers. The black bar represents the position of the probe used to detect the presence of the transgene. Sizes of the fragments (in kbp) obtained after digestion with EcoRI or Asp718 are shown at the bottom. (B) Southern blot analysis of DNA extracted from various tissues. Ten μg of DNA was loaded per lane. Fragment size markers in kbp. (HindIII digest of lamda DNA) are indicated on the left. Lane 1, EcoRI digested human DNA (isolated from blood), lane 2, Eco RI digested DNA from calf #4 isolated from blood; lane 3, Asp718 digested DNA from calf #4 isolated from blood; lane 4, EcoRI digested placental DNA from calf #4; lane 5, Asp718 digested placental DNA from calf #4; lane 6, EcoRI digested DNA from calf #15 isolated from blood; lane 7, Asp718 digested DNA from calf #15 isolated from blood; lane 8, EcoRI digested DNA from calf #15 isolated from ear tissue; lane 9, Asp718 digested DNA from calf #15 isolated from ear tissue; lane 10, EcoRI digested placental DNA from calf #15; lane 11, Asp718 digested placental DNA from calf #15; lane 12, EcoRI digested DNA isolated from the tail of a transgenic mouse harboring the same construct.

mance and ease of calving. For each batch of semen the *'in vitro'* fertilization conditions (heparin concentration and sperm number) were optimized to obtain normal fertilization rates ranging from 50 to 70% as determined by the presence of two pronuclei and a sperm tail. We used either one of two techniques for selection of motile sperm: the swim-up technique[13] and centrifugation through a Percoll gradient (J. Parrish, personal communication). No significant differences in fertilization rates between these methods were recorded. The efficiencies of these and the following steps are shown in Table 1.

Pronuclear injection. In order to visualize pronuclei, fertilized oocytes were centrifuged at 14,500× g for 8 minutes[14]. The time window in which pronuclei could be visualized appeared to be smaller than the period in which murine pronuclei are visible. Cytologically, i.e. after fixation and staining with aceto-orcein, pronuclei are clearly detectable from 16 hours up to 23 hours after fertilization, whereas using interference contrast optics pronuclei can be visualized after centrifugation between 18 hours and 23 hours after fertilization. The number of oocytes in which pronuclei could be visualized during this period was about 10% smaller than the number that was expected based on the cytological data. Possibly, some nuclei are hidden behind the yolk cap that is formed after centrifugation. Pronuclear injection was performed essentially as described[15]. About 10% of the zygotes collapsed after microinjection and were discarded.

Embryo development. After microinjection, the embryos were transferred to microdrops of medium conditioned by bovine oviductal epithelial cells as described[16]. Embryo development was evaluated 9 days after the start of maturation. The development rate varied by about 5% depending on the sperm donor used. In addition, microinjected embryos developed, in general, slower than control embryos that had been centrifuged but not microinjected. The formation of the blastocyst cavity was delayed by approximately 1 day. Also, fewer microinjected embryos developed to the morula/blastocyst stage than control embryos (19% vs. 20–25%, Table 1).

Embryo transfer. The synchronization schedule was set up so that recipients started estrous on the same day at which oocytes were aspirated from slaughterhouse ovaries (i.e. start of maturation is day 1). Recipients received 9-days old embryos, at which time they have developed to the compact morula or early blastocyst stage. These embryos are one day ahead in development compared to the stage of the estrous cycle of the recipients. In case of two microinjection sessions on subsequent days, one group of recipients was used that were in synchrony with the first batch of oocytes collected. Transfers of embryos that developed from oocytes aspirated on the day of the start of estrous gave better results than embryos from oocytes obtained one day later. Due to the somewhat delayed development of microinjected embryos, there appeared to be a better synchrony between the recipients and the first group of embryos. Recipients received two embryos when the quality grade (according to Linder and Wright[17]) was fair to poor and only one single embryo when the quality grade was excellent to good. Each pregnant recipient that received 2 embryos carried only one fetus to term. The overall pregnancy rate was 21%, which is significantly less than the rates reported by others with non-microinjected embryos which had developed *'in vivo'*[18,19]. In the experiments reported here, no transfers with non-injected embryos were performed.

A total of 21 pregnancies were established (confirmed by rectal palpation 45–60 days after transfer). During pregnancy 2 fetuses were lost. One recipient aborted spontaneously for unknown reasons at 7.5 months of gestation. The second fetus, collected at slaughter of the recipient at 3 weeks after the calculated day of parturition, was a full grown dead calf having an abnormal embryonic development called 'schistosoma reflexum'. In both cases no intact DNA could be isolated for analysis. Nineteen calves were born after normal pregnancies. One of these calves died during parturition, and a second, 24 hours after birth, because of pneumonia following accidental

inhalation of milk. A third calf, born after a pregnancy of 10 months and with a body weight of 70kg was euthanized at an age of 3 weeks. Pathological analysis indicated that the animal was suffering from sepsis due to chronic omphalephlebitis. Tissues that could be analyzed from the three dead calves contained no integrated human lactoferrin (hLF) sequences. Therefore, the cause of their death is unlikely to be related to transgene integration. The remaining 16 calves are in excellent health.

Structure of the transgene. The fragment used for injection was designed to express hLF in the mammary gland of a lactating cow. It consists of the coding region of the hLF cDNA[20] fused to the bovine αS_1-casein signal sequence and flanked by the 5′ and 3′ untranslated regions of the bovine αS_1-casein gene. Both untranslated regions are interrupted by an intervening sequence. These introns were included since several groups have shown that the presence of intervening sequences can dramatically increase expression of cDNA-based constructs both in tissue culture and in transgenic animals[21,22].

Expression of the cDNA is controlled by regulatory elements within 15 kbp of 5′-flanking and 6 kbp of 3′-flanking sequences from the αS_1-casein gene. These sequences have been shown to contain elements that are responsible for tissue-specific expression of heterologous genes in transgenic mice[5] (and our own unpublished observations). A schematic drawing of the casein-hLF transgene is shown in Figure 1.

DNA analysis. DNA was isolated from placenta, blood and ear tissue from all calves. Southern blot analysis using hLF cDNA as a probe indicated that in tissues of two calves (#4 and #15) transgene sequences had been integrated into the host genome. Calf #15 (a female) was mosaic for integration of the transgene: placental tissue was positive, whereas in blood and ear tissue no hLF sequences could be detected. The copy number in the placenta was 1–2. The restriction enzyme map of the transgene was different from that expected based on the map of the casein-hLF plasmid (Fig. 1) and based on the pattern obtained in many individual transgenic mice (data not shown). Apparently, a rearrangement had occurred involving a deletion of part of the DNA construct. It is not clear whether this rearrangement event is related to the fact that the transgene could not be detected in all tissues. In mice it has been shown that over 30% of all transgenic animals born are mosaic[23].

Calf #4 (a male) showed, in all three tissues, the same hybridization pattern that was identical to the expected one. Restriction digestions with different enzymes indicated that head-to-tail concatemers of intact copies had integrated and there was no indication of rearrangements. Copy numbers were estimated by comparing the intensities of the transgenic band with bands resulting from hybridization of the hLF probe to human DNA (Fig. 1). In calf #4 between 5 and 10 copies of the transgene had integrated in all three tissues examined.

DISCUSSION

This work proves the technical feasibility of transgenesis in the bovine system: in 2 out of 19 calves born from microinjected zygotes, the introduced DNA was integrated into the host genome. In parallel experiments, the transgenesis rate in mice that received the same casein-hLF construct was about 10% (data not shown). Thus, based on the limited number of animals born, we tentatively conclude that the transgenesis rates in cattle and mice are, in our hands, the same.

Most attempts to produce transgenic cattle have relied partly or totally on *in vivo* procedures[24,25]. Fertilized oocytes were retrieved from superovulated and artificially inseminated cows. Microinjected zygotes were transferred by surgery either directly into the oviduct of recipient cows or into temporary hosts like sheep or rabbits. Obviously, the *'in vivo'* production of zygotes is much more labor intensive than the *'in vitro'* production. Also a larger number of additional animals and veterinary surgeons are involved. Another major disadvantage of the *'in vivo'* procedure is that the stage of pronuclear development at which the zygotes are isolated and processed varies considerably[26], and therefore the most suitable time for microinjections cannot be determined. In addition, up to 50% of the embryos transferred into ligated oviducts in living animals cannot be recovered[27]. Finally, the development of the embryos cannot be followed in the oviduct of temporary hosts, and therefore the stage at which the embryos are recovered is unpredictable. Some of the disadvantages of the *'in vivo'* procedure may be compensated to some extent by the fact that embryos produced by this method have, in general, a better developmental potential[28].

All the disadvantages described above are circumvented when using the *'in vitro'* procedures described here. A large number of aspirated oocytes are matured and fertilized simultaneously and their pronuclei can be injected within a short time. Development of the individual embryos can be monitored closely, which makes it possible to transfer embryos at very specific stages of development. The *'in vitro'* culture of embryos up to the morula/blastocyst stage also allows for additional manipulations such as biopsy of blastomers for transgenesis detection, sexing and cloning. It is expected that the incorporation of these, in part established, technologies in our *'in vitro'* program will further reduce the number of recipients needed to generate (larger numbers of) transgenic cattle.

The procedures described in this paper make use of oocytes obtained from ovaries of slaughtered cows. As a consequence, the genetic background of these animals is unknown. Recent developments in the efficient retrieval of oocytes from live animals using echoscopy[29,30] make it possible to generate transgenic calves that have a defined genetic background both from the maternal and the paternal lineage.

EXPERIMENTAL PROTOCOL

Oocyte source. Ovaries were collected at a local abattoir and transported to the laboratory in a insulated container at 30–32°C. Oocytes, together with follicular fluid, were aspirated from 2–8 mm diameter follicles and pooled into 50 ml conical tubes. Cumulus-oocyte complexes (COC) were allowed to settle into a pellet, after which the supernatant was discarded and the pellet washed in 50 ml TL-Hepes[30]. COC, containing several intact, unexpanded cumulus cell layers, were selected and isolated under a dissecting microscope at 15× magnification, washed four times in 10 ml TL-Hepes, once in 2–3 ml TCM199+10% fetal calf serum (M199)[30] and then transferred to 100 µl droplets of M199 medium under paraffin oil (20 COC/droplet). COC were incubated for 23 h in a humidified atmosphere of 5% CO_2 in air at 39°C.

***'In vitro'* fertilization.** Oocytes were fertilized with frozen thawed-sperm obtained from three different bulls in artificial insemination service. Sperm capacitation was facilitated with heparin[15]. Since sperm from individual bulls respond differently to specific fertilization conditions, semen from each lot was tested in advance to determine optimal heparin and sperm concentration required to maximize normal fertilization frequency and to minimize polyspermy. Fertilization conditions for a given bull were selected after screening at heparin concentrations of 0.0, 1.0 and 10.0mg heparin/ml, and at 1.0, 2.0 and 4.0 × 10^6 motile sperm/ml. Since the proportion of sperm that survives freezing and thawing varies from bull to bull (approximately 30–60% for the bulls used here) sperm preparations were enriched for live, motile sperm by a "swim-up" procedure[15]; alternatively, sperm were centrifuged through a percoll gradient (J. Parrish, personal

communication). After isolation of the motile portion, sperm were counted on a hemocytometer, diluted to an appropriate concentration to yield a 25-fold concentrated stock. The fertilization medium consisted of TALP medium[31] supplemented with 2.0–10.0 mg/ml heparin (from porcine intestinal mucosa, 177 IU/mg; Sigma)[13] and if the cumulus was removed prior to fertilization, 1mM hypotaurine, 10mM penicillamine, 20mM epinephrine and 2mM sodium metabisulfite. Matured COC were selected on the basis of expanded cumulus masses for fertilization, washed once in 10 ml fertilization medium, and either added directly to fertilization droplets, or first stripped of their cumulus investment by gentle pipetting through a small-bore, fire-polished pipet and then added to the droplets. Finally, sperm cells were added to a final concentration of 1×10^6–2.0×10^6/ml. After 16–24 h, presumptive zygotes were removed from fertilization droplets. At this point, 20–30 zygotes for each experiment were fixed in 3:1 ethanol:acetic acid for 24 h, stained with 1% aceto-orcein (in 40% acetic acid), and examined to determine fertilization frequency (percentage of sample with 2 pronuclei and a sperm tail). The remaining oocytes were then prepared for microinjection.

Microinjection. The 26 kbp casein-hLF fragment used for microinjection was released by NotI digestion and purified by agarose gel electrophoresis and electroelution. The final DNA concentration was adjusted to 2.5 μg/ml. Batches of 50 cumulus-intact fertilized oocytes were stripped either as described above or by vortexing 2 minutes in 2ml Tl-hepes medium in a 10ml conical tube. In order to visualize the pronuclei, cumulus free oocytes were centrifuged in 1ml TL-hepes medium 8 minutes at 14,500× g in an Eppendorf centrifuge[14]. Microinjection was performed essentially as described[15].

Embryo culture. Embryos were cultured from the zygote to the compact morula or blastocyst stage in oviductal-tissue conditioned medium[16]. Oviducts were obtained at slaughter and transported at ambient temperature. Luminal tissue from 2–4 oviducts (1–2 cows) was harvested by gently scraping intact oviducts on the outside with a glass slide. The extruded material was washed 5 times in 10 ml TALP-Hepes and diluted in M199 to a tissue:media ratio of 1:50. Media were conditioned in 50 ml "T" flasks containing five ml of oviduct tissue suspension. Conditioned medium was prepared 48 h later from the supernatant after centrifuging tissue suspensions at 13000× g for 10 min., divided into 1.0 ml aliquots and stored at −20°C until used. Conditioned media frequently contained a proteinaceous precipitate after thawing, which was removed by centrifugation. Droplets were covered with paraffin oil and were incubated for 2 h to permit pH to equilibrate prior to adding zygotes. Zygotes were placed in culture droplets within 2 h after microinjection. Initial cleavage (>2 cells) was assessed 42 h after adding sperm. Media were not changed during the course of incubations. Criteria for normal development consisted of attainment of the compact morula or blastocyst stage.

Embryo transfer. Estrous in recipient cattle was synchronized with a 9-day Norgestamet (Intervet, Boxmeer, The Netherlands) treatment (administered in an ear implant according to the manufacturer), and a 500 μg dose of cloprostanol given on day 7 of the Norgestamet treatment. Estrous occurred within 2–3 days after implant removal. Embryos were transferred non-surgically to recipient heifers 5–7 days after estrous (1–2 embryos/uterine horn). Pregnancy was determined by rectal palpation at 45 to 60 days of gestation.

DNA analysis. DNA extraction, Southern blot analysis and hybridization were performed according to standard procedures[32]. The probe used in the Southern blotting experiment was a 758 bp EcoRV-EcoRI fragment covering the 3' part of the hLF cDNA[20].

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6

Large transgenic mammals

G. BREM AND M. MÜLLER

6.1 Introduction

The possibility of expressing foreign genes in mammals by gene transfer has opened new dimensions in the genetic manipulation of animals. The basic techniques of gene transfer were developed in mice, which have been most extensively used in such experiments because they are ideal for studying gene expression during development and for establishing animal models of carcinogenesis and other diseases. Additional applications include analysis of mutations and the use of the transgene as a genetic marker (Jaenisch 1988).

Only a few years after the first successful gene transfer into mice, the new technique was used with farm animals, offering the prospect of completely new breeding strategies and other novel applications. However, despite a decade of gene transfer experiments in farm animals, only a few applications have achieved fruition (reviewed in Wall *et al.* 1992). This is mainly because of fundamental experimental difficulties with these species. In comparison with mice, farm animals have very long generation intervals and the time scale of a transgene project is thus extremely prolonged (Brem 1988, 1989). Naturally this problem is also experienced in conventional breeding programmes, but the major advantage of gene transfer is that significant advances can be made in one generation, whereas conventional breeding techniques require several.

Among the theoretically possible techniques of transferring genes, the only one successfully applied to farm animals so far is microinjection of DNA. Researchers have been rather reluctant to perform gene transfer experiments using retroviral vectors due to the slight risk of recombination with wild-type viruses. The feasibility of the simple

method of using sperm cells as carriers for foreign gene constructs cannot yet be determined (see Section 6.2.4). Without doubt, the most exciting development has been the recent establishment of embryonic stem cells of farm animals and their subsequent use in cloning experiments (Sims & First 1993). Optimization of this technique will give new impetus to gene transfer in farm animals, because it will not only offer the possibility of additive gene transfer and homologous recombination but will also notably reduce such problems as low efficiency, insertional mutations and generation of mosaicism (see Section 6.2.2).

Applications of gene transfer into farm animals fall into three groups:

1. The improvement of production efficiency and quality of animal products
2. The production of new proteins of high value
3. The creation of animal models for human diseases and organs for xenotransplantation

The most obvious application is the optimization of efficient animal production – not only for economic reasons but, more important, for satisfying the ever-growing requirement for food as the world's population increases exponentially. In future, human nutrition will probably include more plant products; therefore, as the amount of agricultural land declines owing to the increase in population, an obvious challenge for gene transfer will be to improve plant productivity.

Certainly by the twenty-first century animal production will be confronted by a dramatic increase in demands for both quality and quantity. These can be met only by intensive efforts in breeding, because the effects of optimizing animal husbandry will presumably soon reach a plateau. Therefore, it is of great importance to improve molecular genetics – for example, gene mapping and gene diagnosis of farm animals. Gene transfer plays an important role in molecular genetics, apart from analytical techniques, because it makes possible the alteration of genotypes. All areas of animal production (e.g., reproduction, health, quantity and quality of products) can be the subject of gene transfer programmes (Table 6.1). So far experiments attempting to fulfil these applications have not been overwhelmingly successful. Nevertheless, it should be possible to use new strategies to improve the efficiency and quality of animal production in the near future, considering the increasing knowledge in genome structure, gene expression and genetic cross-talk.

Table 6.1. *Applications of gene transfer in farm animals*

Improving the efficiency and quality of animal production
Production performance (meat, milk, wool, utilization of nutrients, etc.)
Reproduction performance (pregnancy rate, ratio of new-born to surviving animals)
Health (resistance and susceptibility to diseases, immune response)
Quality of animal products (taste, biological value, etc.)
Processing of animal products (storage, expiring, structure, etc.)
Gene farming
Pharmaceuticals for human and veterinary medicine (vaccines, growth factors, blood coagulating factors, antibodies, etc.)
Raw materials (proteins for further industrial processing)
Enzymes
Nutrients (baby food, diets, geriatric diets)
Animal models and organs
Models of human diseases (high blood pressure, atherosclerosis, cancer, etc.)
Organ donors for xenotransplantation

Gene transfer offers a completely new use for large mammals, that is 'gene farming' (see Section 6.4.2.3). Farm animals can be used to synthesize proteins the production of which has been impossible or difficult to obtain at high purity, as raw materials for industrial processing, pharmaceuticals, enzymes or nutrients.

Another aspect that is potentially of great importance for medicine is the genetic alteration of animal organs so that they can be used for transplantation into humans without being rejected by the recipient (xenotransplantation). This would not only supply an almost infinite number of organs but would also have great significance for transplantation surgery in that the toxic permanent immunosuppression of patients receiving human organs might become obsolete.

Furthermore, farm animals are useful in establishing models for some human diseases; both the causes and potential means of therapy can be investigated in great detail. Of course, this applies only to diseases where animal models with small mammals are adequate (e.g., heart and circulatory diseases, metabolic diseases, transplantation medicine).

In the near future, somatic gene transfer will gain in significance for farm animals. Novel methods for gene transfer into somatic cells (e.g., microbombarding or jet DNA injection of tissue with DNA particles or solutions) promise to be highly efficient. They will provide the possibility of direct genetic immunization (Tang *et al.* 1992) or other

Table 6.2. *Assessment of DNA microinjection for generating transgenic farm animals*

Advantages
Safe and reliable method of transfer
No risk of recombination events with wild-type viruses by avoiding viral vectors
Direct transfer of DNA (cloned gene constructs, YAC or genomic fragments, chromosomes) into (pro-)nuclei
Unlimited size of the DNA transferred
DNA integration usually at a single chromosomal locus
No need for occasionally complicated culture of early embryonic stages
Disadvantages
Technically complicated procedure requiring a range of expensive equipment
Low efficiency combined with high costs
Need for experimenters with high technical skills and extensive experience
Random DNA integration in terms of copy number and chromosomal loci
Necessity of surgical embryo isolation and transfer in certain species

immunomodulations. Because of the thematic frame of this book, this chapter will concentrate on the generation of transgenic farm animals by germ-line integration. A summary of the methods for gene transfer into different species of farm animals will be followed by a description of transgenic species generated so far and their applications.

6.2 Methods for gene transfer into farm animals

6.2.1 DNA microinjection

At present, direct microinjection of DNA into the pronuclei of zygotes is the method of choice for the generation of transgenic livestock (Table 6.2). In principle the procedure does not differ from that used for mice; however, some features of livestock based on distinctions in morphology of the zygotes and in the embryogenesis should be considered. The efficiency of gene transfer is usually notably lower in farm animals than in mice (Table 6.3); this is because of difficulties in isolation and/or transfer of zygotes, microinjection of DNA and insufficient knowledge of the embryology and reproduction of these species.

Since the first reports on the generation of transgenic rabbits, pigs and sheep (Brem *et al.* 1985; Hammer *et al.* 1985) gene transfer into livestock has become a reliable and reasonably efficient procedure.

Table 6.3. *Efficiencies of gene transfer into mammals*

	Mouse	Pig	Sheep	Goat	Cattle
Injectable embryos per donor (*n*)	15	20	5	4	7
Donors per recipient (*n*)	2	2	1	1	1
Pregnancy rate (%)	60	50	60	50	30
Born animals/injected embryos transferred (%)	20	18	25	15	10
Integration frequency (%)	20	10	15	7	7
Efficiency (transgenics/injected embryos transferred) (%)	3	1.5	2.5	1	0.5

Although it is not yet a routine breeding technique, it is used in a number of laboratories. To guarantee the transmission of the foreign gene construct – that is, the integration into somatic cells as well as into the germ-line – the gene transfer has to be carried out as early as possible in the development of the recipient. This requires suitable techniques for the isolation, manipulation and culture of embryonic cells of the corresponding animal species.

A programme for the production of transgenic farm animals consists of six main phases:

1. Cloning of an appropriate gene construct and preparation of DNA solution for microinjection
2. Isolation of embryos, that is, superovulation and insemination of donor animals, and synchronization of recipients
3. Microinjection of DNA solution into pronuclei of zygotes
4. Transfer of injected embryos into recipients
5. Screening of new-born animals for integration and expression of the transferred gene
6. Establishment of (homozygous) transgenic lines by the means of conventional breeding

The different steps required for the production of transgenic livestock and the subsequent breeding tests with the transgenic founder animals are shown in Fig. 6.1.

A typical gene construct should ideally provide all elements controlling specific transcription. The coding portion (structural gene or cDNA) is linked to proximal cis-control elements, the promoter, a

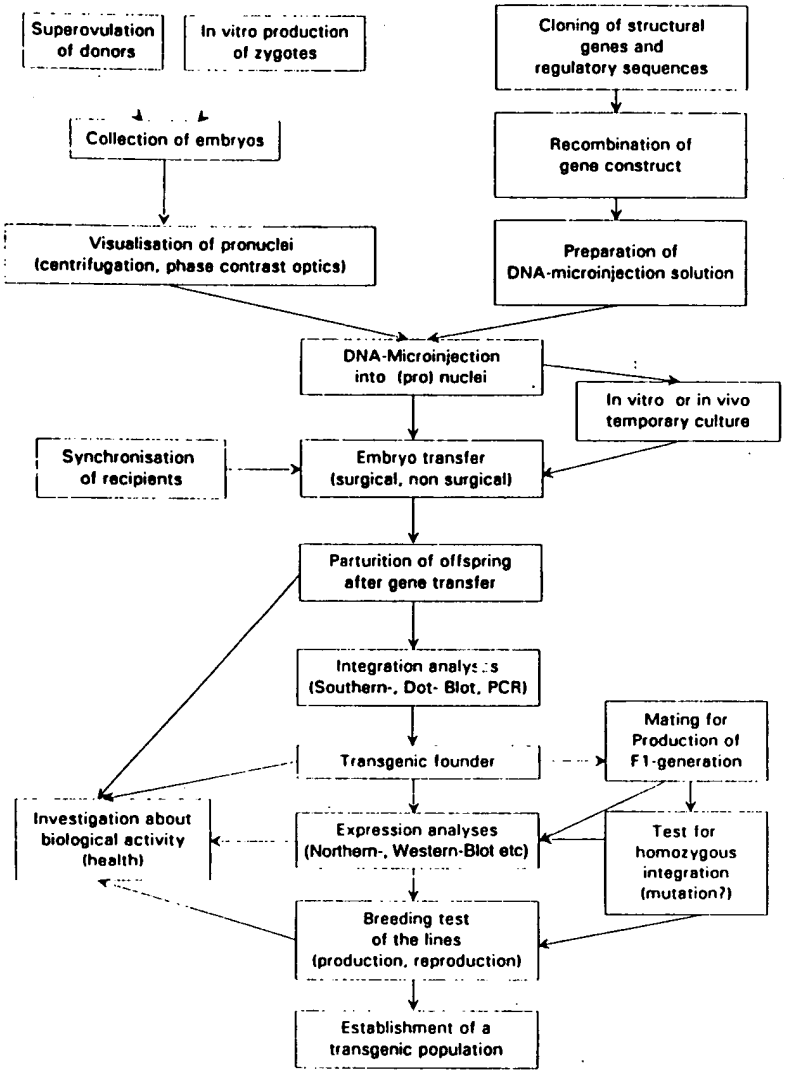


Fig. 6.1. Steps required for the production of transgenic livestock and breeding tests with transgenic animals.

region surrounding the transcription start site specifying quantity, accuracy of initiation and polarity of transcription (for reviews see Mitchell & Tjian 1989; Kozak 1991). More distant cis-elements (Maniatis *et al.* 1987), enhancers (Müller & Schaffner 1990 and references

included) and/or silencers (Goodbourn 1989; Baniahmad *et al.* 1990) control, in co-operation with the promoter, the transcriptional activity and the tissue specificity. Additional remote control elements, referred to as dominant control region or locus control region (LCR), regulate the overall on–off state of the chromatin containing the transcribed unit (reviewed in Eissenberg & Elgin 1991).

The structure of gene constructs depends on the experiment they are designed for, the availability of coding sequences as well as promoter/enhancer elements and the capacity of cloning vectors. Early constructs often originated from vectors designed for transfections and expression in mammalian cells and therefore consisted of a simple transcription unit providing basic transcriptional initiation signals – that is, constitutive promoters and coding sequences (mostly cDNAs) including appropriate transcriptional termination signals. Besides the choice of appropriate promoters, improved knowledge of active chromatin structure and increased experience in generating transgenic animals have helped to define the following crucial properties of gene constructs required for transgene expression (reviewed in Palmiter & Brinster 1986; Rusconi 1991):

1. Removal of all prokaryotic replication vector sequences for avoiding possible inhibitory effects.
2. Inclusion of intron/exon structures if the coding region consists of cDNA. mRNA processing increases the transcriptional activity of transgenes (Brinster *et al.* 1988; Choi *et al.* 1991; Palmiter *et al.* 1991).
3. Addition of an LCR to the gene construct. LCRs of different genes result in high-level transgene expression which is copy number dependent, integration locus independent and tissue specific (Grosveld *et al.* 1987; Bonifer *et al.* 1990; Chamberlain *et al.* 1991; Schedl *et al.* 1993).

There is still a great lack of inducible transcription systems that will permit tight control of gene expression. None of the existing systems guarantees sufficiently low basal levels and a satisfactory induced expression. Moreover, it is difficult to find systems which would not overlap with normal physiological functions. Cloning of additional promoters and further insight into their regulation should help to overcome these problems. A conceivable alternative for controlling transgene expression is to use a heterologous activator system. Two transgenic lines are produced, one harbouring the target transgene

(transresponder), the other an activator transgene (transactivator). The advantage of this multiplex gene regulatory system is that the transgene expression can be activated by mating homozygous lines of transactivators and transresponders (Byrne & Ruddle 1989).

Usually, gene constructs for microinjection are cloned in plasmids, cosmids or λ-phages. These vectors limit the length of the constructs to 20 or 40 kb, respectively. If the transfer of larger fragments is required, different strategies have to be used. The simplest method is co-injection of two or more fragments based on the fact that DNA integration normally occurs at one chromosomal location per genome. The precise mechanism of DNA integration is unknown, although the observed integration pattern indicates the involvement of ligation and/or recombination between the injected fragments.

Microinjection of overlapping fragments which undergo homologous recombination during the integration provides functional transgenes of more than 40 kb length. Pieper *et al.* (1992) injected three fragments spanning the human serum albumin gene (*hSA*) into mouse zygotes. The fragments were 17, 13.1 and 6.7 kb long and had overlaps of 2.5 and 1.85 kb. Twenty (74%) of 27 transgenic mice (DNA integration frequency 25%) had integrated the complete *hSA* locus; thirteen of those mice expressed *hSA*. Therefore, nearly 50% of the transgenic mice showed a functional *hSA* locus resulting from homologous recombination of the injected fragments.

A further possibility for transfer of gene constructs of more than 40 kb is the application of yeast artificial chromosomes (YACs) as cloning vectors. The feasibility of this procedure was demonstrated by Schedl *et al.* (1992). A YAC vector containing the tyrosinase gene (35 kb) was used to generate transgenic mice. The transfer of 147 injected zygotes resulted in 10 transgenic mice (integration frequency 28%). Five mice expressed the expected phenotype. Recently, the same workers successfully transferred even bigger YAC fragments. Schedl *et al.* (1993) have reported the transfer of a 250-kb YAC construct by pronuclear injection of gel-purified YAC DNA. The YAC construct was inserted without major rearrangements, and the transgene expression was integration site independent. Microinjection into all mammals requires the rather complex equipment listed in Table 6.4.

The integration frequency of gene constructs can be positively influenced by the properties of the DNA microinjection solution such as purity and osmolarity (sterile filtered, isotonic), conformation of the gene construct (linearized), as well as its termini (protruding) and

Table 6.4. *Equipment necessary for microinjection*

Equipment	Use
Stereodissecting microscope with transmitted illumination	Embryo collection and preparation for transfer
Stable working bench for micromanipulation	Construction of microinjection unit
Inverse microscope (32×, 400×), Nomarski's optic (and video equipment)	Visualization of (pro-)nuclei and microinjection
Mechanically movable micromanipulator	Holding of fixing pipette
Smooth movable micromanipulator	Moving of injection pipette
Microinjection device	DNA flow in injection pipette
Injection chamber (covered with paraffin) and criss-cross table (temperature-controlled)	Keeping and moving of embryos
Microforge	Production of pipettes
Mechanical pipette puller	Production of injection pipettes
Centrifuge (15,000 *g*)	Removal of particles from the DNA microinjection solution by centrifugation; centrifugation of porcine and bovine oocytes for visualization of the pronuclei

DNA concentration (100 to several 1,000 copies per picolitre). The protocols for the preparation of DNA microinjection solutions used for farm animals are identical to those developed for mice (Hogan *et al.* 1986).

Because gene transfer into farm animals is an extremely complicated procedure, it is necessary to optimize all factors influencing its efficiency (Table 6.5) (Grosschedl *et al.* 1984; Brinster *et al.* 1985; De Pamphelis *et al.* 1988; Jaenisch 1988).

The survival rates of injected embryos and the integration rates achieved in published gene transfer programmes differ among experiments. Success rates in generating transgenic livestock are therefore unpredictable, and in most cases it is impossible to identify the factors that have influenced the gene transfer efficiency. Our knowledge of events during microinjection and DNA integration and of the subsequent development of injected embryos is still too scanty to apply systematic optimization.

Table 6.5. *Factors influencing the efficiency of gene transfer by DNA microinjection*

Factor	Influence
DNA concentration	Optimum integration frequency at concentration of >1 μg/ml, which usually equals several 100 copies/pl; concentrations ≥10 μg/ml might decrease the embryonic survival rate
DNA conformation	Linearized gene constructs integrate with higher frequency than circular DNAs
Ends of the DNA fragments	DNA fragments with protruding ends integrate better than blunt-ending DNA
Injection buffer	DNA solutions are kept in 5–10 m*M* Tris/HCl (pH 7.4)/0.1–0.25 m*M* EDTA; higher EDTA solutions increase embryonic death; alternatively, physiological buffer can be used (48 m*M* K_2HPO_4/4.5 m*M* KH_2PO_4/14 m*M* NaH_2PO_4)
Purity of the DNA solution	Requirement for any particles or components that might obstruct the injection pipette or be embryotoxic
Vector sequences	Transfer of prokaryotic vector sequences might negatively influence the transgene expression level

6.2.2 Embryonic stem cells

Nowadays embryonic stem cells (ES cells) of mice are routinely isolated, cultured, genetically transformed and used for the generation of chimeras in many laboratories (see Chapter 5). So far this method has failed with farm animals due to the lack of appropriate stem cell lines. Many laboratories are devoted to the production of ES cell lines of farm animals. (For cattle see Stringfellow *et al.* 1987, 1991; Schellander *et al.* 1989; Evans *et al.* 1990; Hassan-Hauser *et al.* 1990; Strelchenko *et al.* 1991; Strojek-Baunack *et al.* 1991; Anderson 1992; Saito *et al.* 1992. Swine: Piedrahita *et al.* 1988, 1990a, b; Evans *et al.* 1990; Notarianni *et al.* 1990a, b, 1991; Strojek *et al.* 1990. Sheep: Butler *et al.* 1987; Handyside *et al.* 1987; Rexroad 1990; Meinecke-Tillmann & Meinecke 1991; Notarianni *et al.* 1991. Goat: Meinecke-Tillmann & Meinecke 1991. Rabbit: Giles *et al.* 1993; N. Strelchenko, pers. commun.)

The problems of establishing ES cells of livestock are caused mainly by the limited availability of embryos with a defined genetic background (i.e., there are no comparable inbred strains), and furthermore, the embryonic development of large mammals is not as well understood as that of mice. It is not known at present which developmental stage is optimal for the isolation of stem cells. Cell lines derived from the inner cell mass (ICM) of blastocysts, first, have an extremely slow cell division rate, and, second, tend to differentiate, resulting in cell death after a few passages.

There are several ways to increase the efficiency of establishing ES cells in farm animals. One is to use homologous leukaemia inhibitory factor (LIF) for the inhibition of cellular differentiation. The derivation of ES cells from mouse primordial germ cells has demonstrated the necessity of LIF as well as steel factor (SF) and fibroblast growth factor (Matsui *et al.* 1992). These growth factors are at present being tested on stem cell cultures of farm animals. Other growth factors, specialized media and feeder cell lines are being investigated with the aim of improving the culture conditions. Media include Ham's F20, CMβ (buffalo red liver cell–conditioned), DMEM, BME/Ham's F10 (1:1), TCM-199 and α-MEM (5637-conditioned). The various feeder cell lines as well as mixed cultures include STO fibroblasts, primary embryonic fibroblasts of mice, sheep or swine, bovine foetal fibroblasts derived from liver, kidney, testis and uterus, epithelial cells of oviduct and uterus, granulosa cells and buffalo red liver cells. Growth factors tested besides LIF and SF include ciliary neurotropic factor, insulin, epidermal growth factor, and transforming growth factor beta.

Cell lines similar to stem cells have been isolated from hamster and porcine embryos by Doetschmann *et al.* (1988) and Piedrahita *et al.* (1988), respectively. Evans *et al.* (1990) and Notarianni *et al.* (1990a, b, 1991) reported the *in vitro* culture of cells derived from porcine and bovine embryos for a period of 9 months. These cells are phenotypically ES cells. However, the main property of ES cells (i.e., pluripotency) has yet to be shown. Recently, two groups have succeeded in establishing bovine totipotential cell lines (Sims & First 1993; N. Strelchenko, pers. commun.). N. Strelchenko (pers. commun.) harvested *in vitro*–generated blastocysts on days 8–11 and cultured them on mitosis-inactivated primary mouse embryo fibroblasts in α-MEM medium supplemented with 15% foetal calf serum (FCS). Cells were passaged every 8–12 days. Fifty to sixty per cent of all blastocysts resulted in established cell lines that have so far been cultured for more than 16 passages. These cell lines were used for cloning experiments by means of nuclear transfer into enucleated oocytes. One per cent of the fusion products developed to blastocysts. The cloned embryos were transferred into appropriate recipients (10% pregnancy

rate). Unfortunately, so far all pregnancies have been aborted between days 34 and 45. Sims and First (1993) generated cell lines from *in vitro*–produced embryos of 9–10 days of age (Fig. 6.2). The immunosurgical isolation of the ICM of up to three embryos resulted in stable cell lines with a success rate of 30–50%. The cells were cultured in low-density suspension and kept in CR1 medium supplemented with 5% FCS, additional amino acids, selenium, insulin and transferrin. These ES-resembling cells were used in 659 nuclear transfers into enucleated oocytes (fusion with polyethylene glycol). Seventy per cent of the fused cells underwent cell divisions and 24% of the surviving clones developed to blastocysts. The transfer of 34 blastocysts into 27 cows gave a pregnancy rate of 49%, resulting in the live birth of four healthy calves.

This experiment demonstrated for the first time the feasibility of deriving ES cell lines from embryos, culturing them to totipotency *in vitro* and generating calves via nuclear transfers. Although this procedure has to be repeated and optimized for practical application, it nevertheless marks the beginning of a new era in the generation of transgenic livestock and animal breeding.

The use of ES cells eliminates the majority of problems that have previously occurred in gene transfer experiments:

1. The integration of the transgene can be readily tested in ES cells before further development. Appropriate techniques even make it possible to study transgene expression.
2. Chimeras in cattle can be generated from morulae or blastocysts, which are isolated nonsurgically or produced *in vitro*. After being manipulated, the embryos are transferred by conventional nonsurgical techniques.
3. One hundred per cent of all animals born have integrated the gene construct and have the desired sex.
4. Cloning of ES cells does not result in mosaics. All animals born are genetically identical.
5. Homologous recombination permits the removal of certain existing alleles as well as their replacement with others. This considerably extends the spectrum of gene transfer applications in farm animals.

ES cell–mediated gene transfer will certainly replace DNA microinjection as soon as appropriate cell lines are available for farm animals.

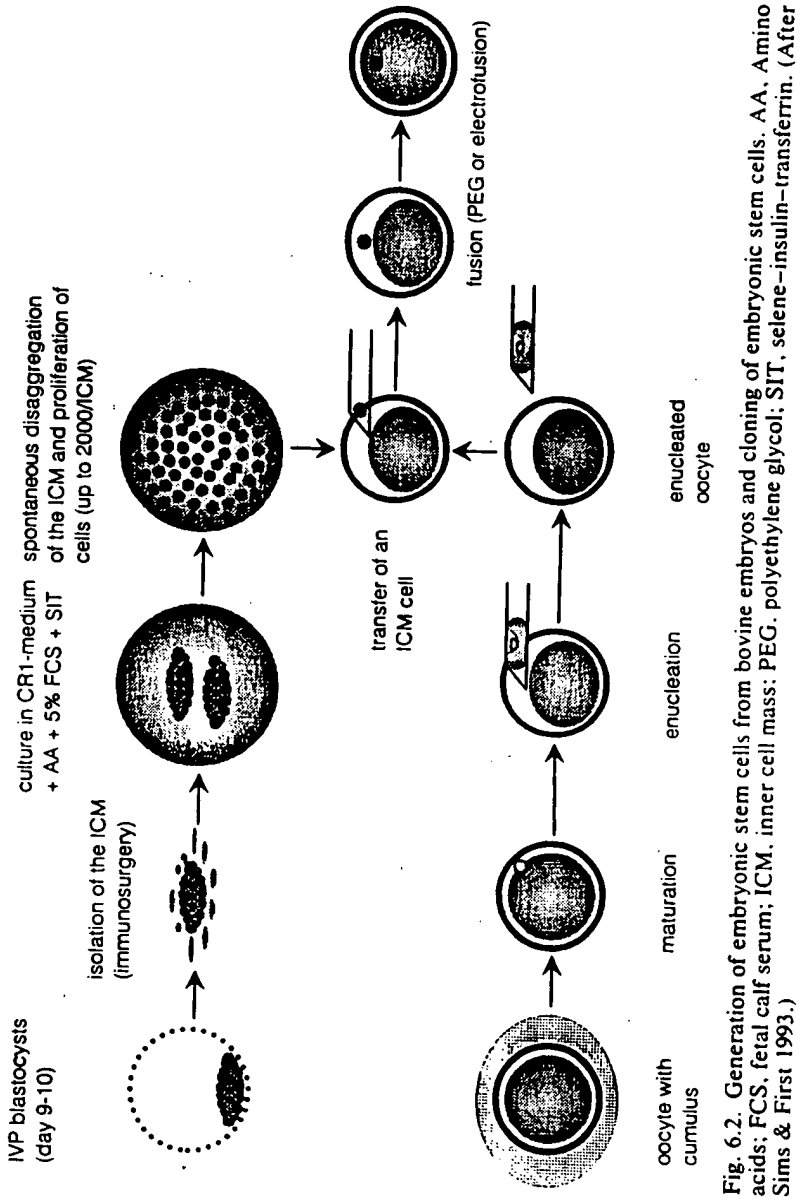


Fig. 6.2. Generation of embryonic stem cells from bovine embryos and cloning of embryonic stem cells. AA, Amino acids; FCS, fetal calf serum; ICM, inner cell mass; PEG, polyethylene glycol; SIT, selene–insulin–transferrin. (After Sims & First 1993.)

6.2.3 *Retroviral vectors*

Viral DNA was first used for gene transfer in 1974; transgenic mice were generated following injection of SV40 DNA into preimplantation mouse blastocysts (Jaenisch 1974; Jaenisch & Mintz 1974). Subsequent experiments were carried out with Moloney murine leukaemia virus. Infection of preimplantation mouse embryos resulted in the integration of viral DNA and its transmission to progeny (Jaenisch *et al.* 1975; Jaenisch 1976).

These early findings resulted in the development of two principal types of vector systems for the expression of foreign genes. The first utilizes replication-competent vectors containing all genes necessary for viral replication. Infection of an early embryo or cell line is followed by reverse transcription of the viral RNA. The newly synthesized proviral DNA containing the transgene is integrated into the host genome. These vectors have the major disadvantage of generating transgenic animals that could potentially produce infectious retrovirus. In addition, they have a limited cloning capacity of only approximately 2 kb. The second system is based on replication-defective retroviral vectors depending on a helper virus cell system for infectivity (Mann *et al.* 1983). Vectors of this kind lack the viral *gag*, *pol*, and *env* genes, but contain the information for encapsulation (ψ^+). Transfection of vector DNA into a helper cell line will produce 'one-round' infectious retrovirus, which inserts the transgene into the genome of infected cells but is subsequently unable to replicate. These vectors have a cloning capacity of approximately 8–10 kb.

Retroviral-mediated gene transfer has been used mainly in mice (reviewed in McLachlin *et al.* 1990a). Since this chapter is concerned predominantly with gene transfer into large mammals, the following is a list of early publications describing gene expression in rodents following retroviral-mediated gene transfer: mutant human dihydrofolate reductase (van der Putten *et al.* 1985); bacterial neomycin gene (Huszar *et al.* 1985; Rubenstein *et al.* 1986); human β-globin gene and neomycin (Soriano *et al.* 1986); *v-myc* and human adenosine deaminase gene and *tk*-neomycin (Stewart *et al.* 1987).

So far, few publications have described the use of retroviral vectors in domestic animals. However, Salter *et al.* (1986, 1987; Salter & Crittenden 1989) achieved the insertion of wild-type and recombinant avian leukaemia virus into the germ-line of chicken by injection of retrovirus into eggs before incubation.

The following initial attempts have been made to use retroviral vectors for gene transfer in sheep and pigs. Harvey *et al.* (1990) injected a high-titer solution of feline leukaemia virus (FeLV) under the zona pellucida of two-to-four-cell ovine embryos. After transfer of the embryos into recipients and 59 days of pregnancy, the foetuses were analysed. Of the 17 foetuses, 2 (12%) showed integrated FeLV-specific sequences. Recent efforts have concentrated on the development of a packaging cell line for FeLV.

Petters *et al.* (1989) established an avian retrovirus (spleen necrosis virus, SNV) in a canine cell line. Approximately 100 cells were injected into 122 porcine blastocysts and transferred to 12 recipients. From the 4 resulting pregnancies 21 normally developed foetuses were isolated after 6 weeks. Polymerase chain reaction (PCR) analysis showed the integration of SNV DNA in the organs of 17 foetuses (80%).

There are some obvious advantages of using retroviral vectors, including the fact that infection generally results in collinear integration of few gene copies, many cells can be handled simultaneously and the host genome is usually unaffected by the integration of the retroviral sequences. However, some major disadvantages have become apparent. Expression studies in transgenic mice generated by retroviral vectors revealed a frequent lack of transgene transcription controlled by retroviral enhancer/promoters. Apparently there are mechanisms in early embryos or ES cells that abolish the expression of these vectors during development and occasionally permit weak expression in adults. Although DNA methylation was initially thought to be responsible, it does not seem to be the only cause, as the degree of methylation is not sufficiently correlated to the expression data. However, this problem can be avoided by the use of appropriate 'external' promoter elements instead of the retroviral expression cassette. As mentioned earlier, retroviral vectors have a limited cloning capacity (maximum of 8–10 kb).

Safety considerations are very important with the use of viruses as gene transfer agents (Goff & Shenk 1993). The potential of spreading infectious vector virus is extremely unlikely with replication-defective retroviral vectors. These 'one-round' infectious vectors are generally used in gene transfer experiments. However, even replication-defective retrovirus might cause the activation of cellular retroviral sequences by recombination, though the occurrence of such an event is highly unlikely. Nevertheless, mouse models should be used in long-term experiments to transmit retroviral transgenes through many generations with the aim of satisfying safety demands. Perpetual improvement of retroviral vector systems will guarantee high safety stan-

dards that should rule out any hypothetical risks (Dougherty & Temin 1987; Salmons *et al.* 1991; reviewed in Boris-Lawrie & Temin 1993).

Our laboratory constructed a retroviral vector system lacking all infectious properties (Janka *et al.* 1993), so as to avoid the potential risk of retroviral recombination and yet retain the advantages of the retroviral integration characteristics. The system is based on a vector containing the packaging signal (BAG vector) and a cell line (GAP_p BAG) providing only the intracellular predecessors of virus particles – that is, core particles. GAP_pBAG lacks the *env* gene and therefore is unable to produce infectious retrovirus. Core particles can be isolated either from cytoplasmic extracts or from medium supernatants and subsequently injected into nuclei of embryos.

6.2.4 Alternative gene transfer techniques

Alternative methods of gene transfer are provided by techniques originally developed for the transfection of eukaryotic cells. However, DNA transfection procedures using calcium phosphate, DEAE-dextran or electroporation are inappropriate because of their relative inefficiency and problems with unstable integration and rearrangements. The use of liposome-mediated gene transfer is combined with blastocyst manipulations. Rottmann *et al.* (1985) microinjected liposomes containing gene constructs into 89 mouse embryos. Five of the resulting 24 animals were transgenic. Reed *et al.* (1988) performed a similar experiment with bovine blastocysts, though the results have not been published.

A stunningly simple procedure for generating transgenic animals has been published by Lavitrano *et al.* (1989). DNA (pSV–CAT gene construct) was mixed with spermatozoa before *in vitro* fertilization of mice. Nearly 30% of the new-born mice carried the foreign DNA and transmitted it to their progeny. Expression of the pSV–CAT construct could be monitored. The same gene construct was used for spermatozoa-mediated gene transfer into pigs. The surgical insemination of 22 sows resulted in 16 pregnancies. Ten piglets (21% of newborn piglets) were transgenic and expressed the SV–CAT construct (Gandolfi *et al.* 1989).

Brackett *et al.* had already demonstrated in 1971 that sperm cells could function as DNA transport vehicles into rabbit oocytes. However, the SV40 DNA used was not shown to be integrated into the host genome.

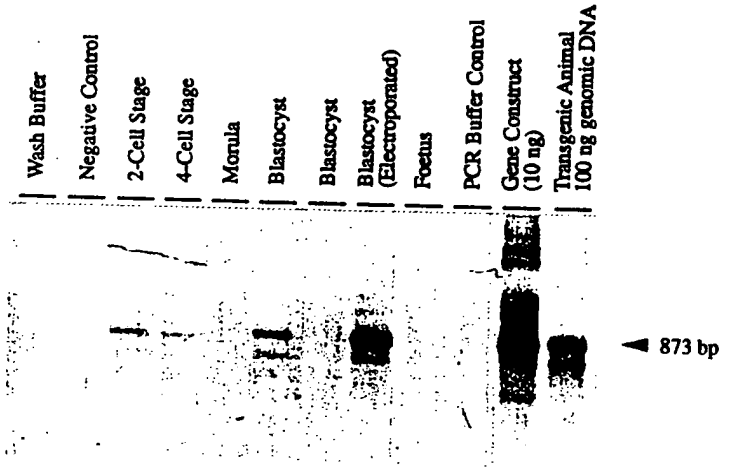


Fig. 6.3. Polymerase chain reaction (PCR) and Southern blot analysis of murine embryos and foetuses generated by *in vitro* fertilization with sperm cells incubated with foreign DNA.

The great potential of spermatozoa-mediated gene transfer led to immediate efforts by various laboratories to repeat the experiments just described. So far, no successful genetic transformation by this method has been reported (Brinster *et al.* 1989). PCR analysis has been used to trace exogenous DNA being carried into oocytes by sperm cells. Hochi *et al.* (1990) found that the last embryonic stage at which they could detect the foreign DNA was the blastocysts. Our own experiments showed specific amplification of a foreign gene construct (MT–GHRH) in early mouse embryos, but no exogenous DNA was detectable after 10 days of development (Fig. 6.3).

The mechanisms of spermatozoa-mediated gene transfer have to be explored further, before its use becomes a practical possibility.

6.3 Gene transfer into pigs

6.3.1 Treatment of donors and recipients

Most gene transfer experiments in livestock have been performed with swine. This is due in part to the economical importance of pig production in many countries and in part to the reproductive nature of pigs. Superovulation of sows usually results in more than 20 embryos for microinjection per donor (Table 6.6). Embryos can be collected not

Table 6.6. *Gene transfer programmes in pigs: treatment of donors and isolation of embryos*

Breed	Age/ body weight	Synchronization	Superovulation (PMSG)	Induction of ovulation (HCG) [hr after PMSG]	Insemination, hr after HCG	Reference
German Landrace, Pietrain, cross-breeds	Prepuberal gilts/60–90 kg	Not necessary	1,250 IU	750 IU i.m. [72 hr]	24 + 36 hr	Brem *et al.* (1985)
	Mature gilts	15 mg Altrogenost for 5–9 days starting at 12th–16th day of cyclus	1,500–2,000 IU 24–30 hr after last admin. of Altrogenost	500 IU [72 hr]	18 + 36 hr	Hammer *et al.* (1985); Miller *et al.* (1989)
	Mature gilts	120 mg Methallibure over period of 6 days, starting at 10th–15th day of cyclus	1,500 IU s.c., 24 hr after last admin. of Methallibure	500 IU i.m. [72 hr]	16 + 36 hr	Pursel *et al.* (1988)
Large White	Multiparous		750 IU	500 IU [44 hr]	Mating	Vize *et al.* (1988)
Yorkshire	—	15 mg Altrogenost for 9 days, starting at 12th–16th day of cyclus	2,000 IU s.c., 24 hr after last admin. of Altrogenost	1,000 IU [78 hr]	24, 36 + 48 hr	Ebert *et al.* (1988)
Cross-breeds, Landrace, Yorkshire, Hampshire	Sows		400 IU i.m.	200 IU	24 + 30 hr after begining of oestrus	Wieghart *et al.* (1990)
	8.5–9 months/ 115–25 kg	5 g Suisynchron-Premix over a period of 15 days	1,500 IU	500 IU [79 hr]	24 + 39 hr	Brüssow *et al.* (1990, 1991)
Large White, Landrace, crosses	230 days	Cloprostenol	1,000 IU	500 IU [72 hr]	32 hr	French (1991)
	>7 months	Allyl-Trenbolone over a period of 12–14 days, 15 mg/day oral, on last day 2 i.m. admin. of 10 mg $PGF_{2\alpha}$ in an 8-hr interval	1,500 IU i.m. after 24 hr	750 IU [80 hr]	12 + 24 hr after beginning of oestrus	Swanson *et al.* (1992)

only from living donors by surgical flushing of oviducts but also from slaughtered animals. This greatly increases the number of potential donors and therefore the number of available embryos. While both quantity and quality of embryos isolated from prepuberal donors are higher than from older animals, multiparous sows offer notable advantages as recipients in terms of pregnancy rates and embryonic survival. The use of older sows requires the synchronization of the cyclic hormonal activity by medications that are unlicensed in some countries (e.g., Federal Republic of Germany).

Our experimental procedure, described in the following paragraphs, is therefore designed for prepubescent gilts. Superovulation in donor animals is stimulated on day 0 by administration of pregnant mare's serum gonadotropin (PMSG) (1.250 IU, Intergonan, VeMie). Ovulation is induced by human chorionic gonadotropin (HCG) (750 IU, Ekluton, VeMie) on day 2 followed by two consecutive inseminations after 24 and 36 hr. The fertilized oocytes are collected 24 hr later (Table 6.7). The recipients are treated likewise, except with a 12-hr delay and a reduction in the dosage of PMSG treatment to 750 IU. Manipulated embryos are transferred to the recipients 60–3 hr after induction of ovulation.

Embryos are isolated surgically from anaesthetized donors (160 mg azaperon, Stresnil, Janssen per 400 mg metomidat hypochloride, Hypnodil, Janssen). The operation is carried out on a mobile operating table with the animal lying on its back. After the usual hygienic precautions, the abdomen is opened in the linea alba, and uterus, oviduct and ovary are retracted from the abdomen. The oviduct is reached via perforation of the uterus at the uterotubular border with a curved glass canula and is flushed with 50 ml phosphate-buffered Dulbecco's medium (PBS) (Table 6.7), which is collected in a sterile petri dish (Fig. 6.4). The zygotes are isolated, cultured in fresh medium and classified morphologically with the help of a stereomicroscope.

The number of isolated zygotes suitable for microinjection was positively correlated with the length of the seasonal signs shown by the donors as well as by their body weight (Table 6.8). In addition, we observed an increase in fertilized oocytes by using different boars for the insemination of the donor animals. The influence of stress factors (e.g., transport, centrifugation and microinjection) on the further development of transferred zygotes is summarized in Table 6.9.

Table 6.7. *Isolation of embryos from superovulated sows*

Hr after HCG	Medium	Centrifugation	Reference
60–3	50 ml PBS (Dulbecco's) per oviduct	15,000 *g*, 3 min	Brem *et al.* (1985)
57–60	PBS + 0.4% BSA	—	Pursel *et al.* (1988)
10/ovul.	—	7,000 *g*, 3 min	Vize *et al.* (1988)
	10–15 ml Kreb's Ringer–biscarbonate solution	13,000 *g*	Ebert *et al.* (1988)
52–4	—	—	Polge *et al.* (1989)
	BMOC-3 buffered with Hepes	10,000 *g*	Wieghart *et al.* (1990)
59–66	—	5,000 *g*, 6 min	Swanson *et al.* (1992)
65–8	PBS	9,800 *g*, 6–10 min	Brüssow *et al.* (1990, 1991)

Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; $BMOC^{-3}$, modified Brinsters mice ova culture medium 3.

Fig. 6.4. Surgical embryo collection by flushing of a porcine oviduct.

Table 6.8. *Influence of donor's body weight on number of collected and microinjected porcine oocytes*

Body weight (kg)	Donor (*n*)	Oocytes per donor animal: Isolated[a] (*n*)	Microinjected: *n*	Microinjected: %
≤ 60	35	30.7	20.1	66
61–70	221	34.6	20.0	58
71–80	182	34.2	18.6	54
≥81	51	38.1	17.2	45
Total	489	34.5	19.2	56

Note: 509 stimulations; 489 oviduct flushings (96%).
[a] Embryo collection rate (collected oocytes per corpora lutea), 95%.

Table 6.9. *Influence of centrifugation and microinjection on the developmental rate of porcine zygotes kept in oviduct* in vivo *culture for 4 days*

Manipulation of zygotes	Transfers in recipients (*n*)	Embryos transferred into oviduct (*n*)	Collection rate (%)	Developmental rate (%)
No transport, no microinjection	14	655	73	52
Centrifugation	12	639	66	44
Centrifugation and transport	4	185	54	30
Centrifugation and microinjection	25	1,147	68	14
Centrifugation, microinjection and transport	13	554	64	10

The pronuclei of porcine zygotes are obscured microscopically, because their cytoplasm is extremely granular (Fig. 6.5). Therefore, before DNA microinjection, the pronuclei have to be visualized by centrifugation of the zygotes (e.g., 15,000 *g*, 3 min; Table 6.7) (Wall *et al.* 1985). After culturing of the microinjected cells for a couple of hours, morphologically damaged cells are removed.

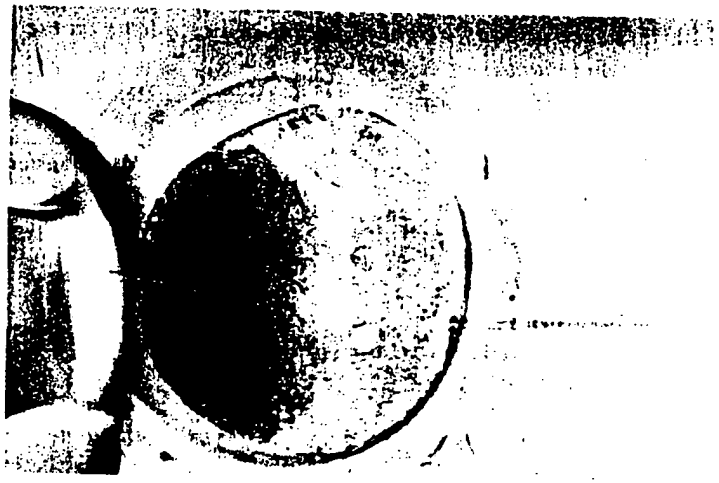


Fig. 6.5. DNA microinjection into the pronucleus of a porcine zygote after centrifugation.

Embryos were transferred into the oviduct of 12-hr asynchronous recipients. Surgical preparation of these animals was performed in the same manner as for the donors. Migration of embryos ('spacing') results in their equal distribution in both uterine horns.

Under appropriate conditions, porcine zygotes can be cultured *in vitro* to a developmental stage that enables transfer directly into the uterus. This procedure is obligate for nonsurgical embryo transfer, first applied by transcervical implantation (Polge & Day 1968; Sims & First 1987). Our experiments applying nonsurgical transfer in swine resulted in pregnancy rates of 10% (Reichenbach *et al.* 1993) and 50% respectively (J. Mödl *et al.* unpublished data). However, the embryo survival rate in these experiments was considerably lower than after surgical transfer.

Pregnancy and embryo survival rates (embryo transfer efficiency) are positively influenced by the intensity of oestrus and the recipients having an optimum body weight (70–80 kg) (Table 6.10). Pursel *et al.* (1987) and Wei *et al.* (1993) noticed that efficiency was increased from 0.83% to 1.04% by co-transferring control embryos alongside the injected embryos. Similar results were obtained in our experiments (Table 6.11). Furthermore, a modified embryo transfer programme

Table 6.10. *Influence of body weight of prepuberal recipients on the pregnancy rate and number of piglets*

Body weight (kg)	No. of transfers	Pregnancy rate (%)	Piglets per	
			Litter (*n*)	Transfer (*n*)
≤70	48	31	3.4	1.1
71–80	92	48	4.0	1.9
≥81	57	30	5.4	1.7
Total	197	37	4.3	1.6

Table 6.11. *Influence of transferring control embryos on the gene transfer efficiency in pigs*

	Transfer of microinjected zygotes	
	Without control embryos	With control embryos
No. of transfers	20	11
Microinjected embryos per recipient (*n*)	38	28
Control embryos per recipient (*n*)	—	8
Pregnancy rate (%)	40	64
Total no. of new-born piglets	38	37
Embryonic survival rate (%)	5	9.3
Transgenic piglets	4	3
Integration rate (%)	10.5	8.1
Efficiency (%) (transgenic piglets per embryos transferred	0.5	1.0

has been developed to reduce the number of animals and surgical manipulations required (Table 6.12).

Although all criteria affecting the integration and developmental rate of transferred embryos have not been fully clarified, the data collected in other laboratories indicate tendencies similar to those mentioned here (Hammer *et al.* 1986; Pursel *et al.* 1987, 1988; Rexroad *et al.* 1987).

Table 6.12. *Usage of donor animals as recipients during the same embryo transfer experiment*

Treatment	Donors (*n*)	Embryos per animal (*n*)	Pregnancy rate (%)	Foetuses and/or piglets per		Survival rate (%)
				Litter (*n*)	Donor/recipient (*n*)	
Insemination of donors during the following oestrus after embryo collection	14	—	43	10.7	4.6	—
Transfer of microinjected embryos into synchronized recipients (conventional)	42	36	40	4.8	1.9	5.3
Transfer of unmanipulated embryos into oviducts of donors	30	24	53	6.3	3.3	14.2
Transfer of microinjected embryos into oviducts of donors	45	35	40	3.9	1.6	4.5

6.3.2 *Transgenic pigs*

The use of 21 different constructs in gene transfer experiments revealed the following data: embryonic survival rate, 7.8%; integration rate of the gene construct, 8.7%; expression rate among transgenics, 42%; and total efficiency (transgenic piglets per transferred microinjected embryos), 0.67% on average (Table 6.13).

Most gene transfer experiments have been carried out for the purpose of altering growth performance and/or carcass composition. In addition, some transfer programmes have concentrated on the improvement of disease resistance, expression of foreign proteins in the mammary gland and production of functional human haemoglobin.

6.3.2.1 *Gene constructs altering growth-related functions*

Experimenters attempting to change growth performance by transgenic means have to be aware that growth is a complex process depending on both genetic and environmental factors. Briefly, the growth hormone cascade consists of polypeptides synthesized in the hypothalamus, that is, growth hormone–releasing hormone (GHRH) and its antagonist, controlling the production of growth hormone (GH) in the pituitary gland. Association of GH with its receptor induces synthesis of insulin-like growth factor I (IGF-1). All of these hormones are tightly controlled by positive and negative feedback involving both themselves and others.

Despite positive effects of GH administration in livestock and in contrast to the findings in transgenic mice, GH-transgenic pigs did not generally demonstrate increased growth performance (Hammer *et al.* 1985; Ebert *et al.* 1988; Viz *et al.* 1988). This is caused by a 20% reduction in their food uptake combined with an increased utilization of nutrients. Only after the application of a protein-enriched diet supplemented with lysine, minerals and vitamins did GH-transgenic pigs attain a 15% higher daily weight gain than control animals (Pursel *et al.* 1988, 1989a). In terms of production-relevant properties, the transgenic pigs showed a massive reduction of the back fat thickness (40%) (Hammer *et al.* 1986; Pursel *et al.* 1989a, 1990).

However, overexpression of GH by use of strong promoters (i.e., metallothionein promoter or M-MLV promoter/enhancer) causes a variety of pathological side effects in pigs, such as gastric ulcers, severe synovitis, dermatitis, nephritis, cardiomegaly, pneumonia, in-

Table 6.13. *Results of transferring gene constructs into pigs*

Construct	Embryos injected and transferred (*n*)	Offspring (incl. foetuses)		Integration		Efficiency (%)	Expression		Reference
		n	%	*n*	%		*n*	%	
mMT-I-hGH	2,035	192	9.4	20	11.0	0.98	11/18	61	Hammer *et al.* (1985)
mMT-I-hGH	268	15	5.6	1	6.7	0.4	—	—	Brem *et al.* (1985)
mMT-I-hGH	1,014	21	2.1	4	19	0.4	2/4	50	Brem *et al.* (1988b)
mMT-I-bGH	2,193	149	6.7	11	7.4	0.5	8/11	73	Rexroad and Wall (1987)
hMT-pGH	423	17	4.0	6	35.3	1.42	1/6	17	Vize *et al.* (1988)
MLV-rGH	170	15	8.8	1	6.7	0.59	1/1	100	Ebert *et al.* (1988)
bPRL-bGH	289	20	6.9	5	25.0	1.73	3/3	100	Polge *et al.* (1989)
WAP-2-hGH	1,028	51	5.0	7	13.7	0.7	1/3	33	Brem *et al.* (1989)
mMT-I-hGRH	1,041	54	5.4	6	0.6	0.6	1/4	25	Brem *et al.* (1988a,b)
mMT-I-hGRH	2,236	177	7.9	7	4.0	0.3	2/7	29	Pursel *et al.* (1989b)
MT-hGRF	2,627	238	8.9	8	3.4	0.30	2/8	25	Pursel *et al.* (1989b)
Alb-hGRF	968	108	11.2	5	3.8	0.52	3/3	100	Pinkert *et al.* (1987), Pursel *et al.* (1989a)
mMT-IhIGF1	387	34	8.8	4	11.8	1.03	1/4	25	Pursel *et al.* (1989a)
mMT-Mx	1,083	22	2.0	6	27.3	0.6	0	0	Brem *et al.* (1988a, b), Müller *et al.* (1992)
SV-Mx	809	26	3.2	1	3.8	0.1	—	—	Brem *et al.* (1988b), Müller *et al.* (1992)
mMx-Mx	1,629	77	4.7	8	10.4	0.5	2/5	40	Brem *et al.* (1989), Müller *et al.* (1992)
PEPCK-bGH	1,057	124	11.7	7	5.6	0.66	5/7	71	Wieghart *et al.* (1990)
MLV-pGH	410	59	14	6	10	1.5	1/6	17	Ebert *et al.* (1990)
CMV-pGHSV 40	372	32	9	15	47	4.0	2/23	15	Ebert *et al.* (1990)
MLV-pGH-SV 40	312	33	11	10	30	3.2	0/10	0	Ebert *et al.* (1990)
k-γ-l-mAB	—	—	—	2	—	—	1/1	100	Weidle *et al.* (1991)
WAP-WAP	850	189	22.2	5	2.6	0.59	—	—	Wall *et al.* (1991)
IgA	542	—	—	2	—	0.4	2/2	100	Lo *et al.* (1991)
LTRcSKI	1,091	302	28	29	9.6	2.7	10/29	34	Pursel *et al.* (1992)
LCR-αaβ	709	112	15.8	3	2.6	0.42	3/3	100	Swanson *et al.* (1992)
Total	26.602	2,067	7.8	179	8.7	0.67	62/148	42	

sulin resistance and reduced fertility (Pursel *et al.* 1989a; Ebert *et al.* 1990). Gene constructs with different regulatory elements providing lower constitutive (PEPCK promoter) or artificially inducible levels of GH expression (prolactin promoter) resulted in the desired increase in carcass leanness and reduction in detrimental side effects (Polge *et al.* 1989; Wieghart *et al.* 1990).

Pursel *et al.* (1992) transferred the avian *c-ski* gene controlled by the mouse sarcoma virus promoter/enhancer for the purpose of improving muscle development. The proto-oncogene *c-ski* induces myogenic differentiation. As shown in mice (Sutrave *et al.* 1990), the *c-ski* gene construct was predominantly expressed in skeletal muscles, causing selective hypotrophy of type II fast fibers. The extent of this was highly dependent on the transgene expression level. Five transgenic pigs showed muscle hypertrophy at the age of 3–7 months. However, five other pigs developed muscle atony between shortly after birth and 3 months. Histological examination demonstrated a high degree of vacuolic degeneration of the muscle tissue.

Altering growth-related traits by gene transfers requires gene constructs that ideally show gene expression correlated to specific metabolic procedures or inducible by administered inducers. This prevents detrimental overexpression and guarantees transgene activity limited to growth-performance-related periods.

6.3.2.2 *Gene constructs for improving disease resistance*

An important and challenging aspect of gene transfer in farm animals is the introduction of beneficial genes to improve health and disease resistance (Müller & Brem 1991; Staeheli 1991). Such attempts in pigs include the use of immunoglobulin gene constructs ('genetic immunization') (Lo *et al.* 1991; Weidle *et al.* 1991) and the transfer of a murine gene (*Mx1*) conferring specific resistance to influenza viruses (Müller *et al.* 1992).

Expression of a transgenic immunoglobulin specific for a common pathogen could provide an animal with congenital immunity for that pathogen. As shown by many investigations, cloned genes coding for monoclonal antibodies can be expressed in large amounts in transgenic mice. These mice produce antibodies against specific antigens without prior contact or immunization (reviewed in Storb 1987; Bluethmann 1991; Iglesias 1991). To evaluate whether antibodies of diagnostic or

therapeutic interest could be produced in farm animals, Weidle *et al.* (1991) introduced the genes for the light and heavy chain of a mouse monoclonal antibody directed against 4-hydroxy-3-nitrophenylate into the germ-line of pigs. One pig expressed the transgene. A level of 1,000 μg monoclonal antibody per millilitre was measured in the serum. Isoelectric focusing experiments revealed that in the transgenic pig only a minority of the bands matched those of the purified mouse antibody. This finding can be explained by assuming tissue-specific post-translational modifications and heterologous immunoglobulin chain associations. Since the production of antibody to various polysaccharide antigens can be protective against pathogenic bacteria, Lo *et al.* (1991) generated transgenic pigs carrying genes coding for the mouse α- and κ-chains for antibodies against phosphorylcholine. Two transgenic pig lines were established; in both founder animals, only the mouse IgA transgene was integrated. Despite the absence of any mouse L-chain, high titers (600 to more than 1,000 μg/ml) of mouse IgA were found in the serum, indicating that it was able to form complexes with the endogenous pig L-chain. Little if any of the mouse IgA in the porcine serum was capable of binding phosphorylcholine specifically, but it is suggested that the mouse IgA may be able to function as an antigen receptor in pig B-cells and therefore contribute to the antibody repertoire of the transgenic animal. Despite some unexpected findings, both experiments illustrate the potential of introduction of beneficial traits such as germ-line-encoded immunity into pigs.

In animals only a few instances of a single genetic locus responsible for disease resistance are known. A well-examined example is the Mx1 gene product of certain mouse strains. The synthesis of mouse Mx1 protein in various cell lines and transgenic mice demonstrated that it is both necessary and sufficient to promote resistance to influenza viruses in previously susceptible cells and animals (Staeheli *et al.* 1986; Arnheiter *et al.* 1990; Kolb *et al.* 1992). With the cloning and functional characterization of this specific disease-resistance gene, it was possible to undertake a gene transfer programme examining whether *Mx1* transgenic pigs would show reduced susceptibility to influenza infections (Müller *et al.* 1992). The gene construct used consisted of the *Mx1* cDNA controlled by the *Mx1* specific regulatory elements, which is inducible by interferons. Five transgenic pig lines were established, of which two showed interferon-inducible expres-

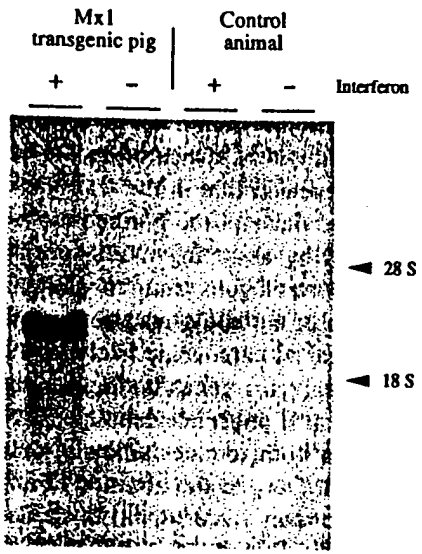


Fig. 6.6. Northern blot analysis of *Mx1* transgenic pigs.

sion of transgene-specific mRNA (Fig. 6.6). Despite extensive protein analysis, no mouse Mx1 protein could be detected in the transgenic animals.

The most likely explanation for this result is based on the findings that permanent high-level expression of Mx1 protein, particularly its presence during embryogenesis, is not tolerated by the organism (Arnheiter *et al.* 1990; Kolb *et al.* 1992; Müller *et al.* 1992). Although the gene construct was shown to be tightly regulated in mouse cell lines, it showed a basal low-level transcriptional activity in tissues of the transgenic animals. Considering the deleterious effect of permanent Mx synthesis, the observed leakiness of the promoter used may have allowed the embryonic development of only those animals with abolished translation of the gene construct. The gene transfer experiments in pigs demonstrated that the choice of the regulatory elements controlling *Mx1* transgene expression is crucial. Therefore, future gene constructs should guarantee tight transgene regulation during embryonic development and highly inducible Mx1 synthesis after exogenous stimuli (Staeheli 1991; Müller *et al.* 1992).

6.3.2.3 Transgene expression in the mammary gland

It is obviously more efficient to obtain milk from a ruminant than from a pig; hence, the conversion of mammary glands of transgenic animals into bioreactors is more frequently attempted in these species (reviewed in Hennighausen, 1992; see Sections 6.4, 6.5 and 6.6). Nevertheless, due to greater experience in generating transgenic pigs, they have been used to establish a transgenic domestic animal system for targeting the synthesis of foreign protein to the mammary gland. Two laboratories reported the high-level synthesis of heterologous protein in the mammary gland of swine (Wall *et al.* 1991; Velander *et al.* 1992). In both cases the regulatory sequences controlling the gene of interest were derived from the mouse whey acidic protein (WAP) (Campbell *et al.* 1984). WAP gene constructs have been used successfully to direct synthesis of foreign proteins to the mammary glands of mice (Andres *et al.* 1988; Pittius *et al.* 1988).

Wall *et al.* (1991) generated three transgenic pig lines expressing mouse WAP. Transgene expression was found only in the mammary glands up to a level of 1 g/l. However, three further transgenic lines showed dramatically reduced lactation caused by undifferentiated mammary gland tissue. The phenomenon of 'milklessness' has been previously observed in WAP transgenic mice (Burdon *et al.* 1991). There is evidence that early WAP expression in virgin pigs inhibits the functional development of the mammary gland (Shamay *et al.* 1992).

In a second experiment a fusion gene consisting of the cDNA for human protein C *(hPC)* inserted in the first exon of the mouse WAP gene was inserted into the germ-line of pigs (Velander *et al.* 1992). *hPC* is a regulator of hemostasis, suggesting its potential therapeutic use for many disease states. Two transgenic pig lines showed high-level expression of *hPC* (highest 1 g/l). Protein C produced in transgenic pig milk possessed anticoagulant activity equivalent to that of *hPC* derived from human plasma.

6.3.2.4 Production of human haemoglobin

Transfusion-related diseases, combined with shortages and difficulties in long-term storage of blood, make an alternative to human erythrocytes as a source of haemoglobin desirable. The synthesis of human haemoglobin in the serum of transgenic pigs is an approach yielding

appropriately modified cell-free solutions of haemoglobin that can be used as a source of an oxygen-carrying erythrocyte substitute. Swanson *et al.* (1992) used a construct consisting of the LCR from the human β-globin locus and two copies of the human α_1- and one copy of the human β^A-gene to obtain three transgenic pigs. All transgenic animals expressed the human genes at a copy-number-dependent level. They were healthy and nonanaemic, and grew at rates similar to those of nontransgenic control animals. Purification of the human haemoglobin from porcine proteins including porcine haemoglobin was accomplished by ion-exchange chromatography. The human haemoglobin derived from transgenic pigs and that from human serum exhibited similar oxygen equilibrium curves.

6.4 Gene transfer into sheep

Most gene transfer experiments in ruminants have been carried out with sheep. This is due mainly to the relatively short generation intervals of this species and to financial considerations.

6.4.1 Treatment of donors and recipients

The isolation of a sufficient number of embryos for microinjection requires the precise control of oestrus and ovulation of the donor animals. A variety of protocols are used to collect eggs from sheep, although no consensus has emerged on an optimal procedure. Sheep are seasonally anoestrus (as are goats); therefore, the effectiveness of superovulation varies with season. Synchronization is usually achieved by the intravaginal administration of progestagen depots (i.e., sponges) for 10–16 days. Superovulation is induced by administration of follicle cell–stimulating hormone (FSH) or PMSG during the late luteal phase of the oestrus (Table 6.14), followed by artificial or natural insemination of sheep 16–24 hr later. After a further 16–26 hr, the zygotes are isolated by obligatory surgical techniques. On average, superovulation of sheep yielded 7.2–10.5 zygotes per donor (Hammer *et al.* 1986; Rexroad & Pursel 1988; Halter *et al.* 1993). Approximately 50% of the isolated embryos could be used for microinjection of DNA.

The pronuclei of fertilized ovine oocytes are visible under interference-contrast microscopy (Fig. 6.7) without prior centrifugation (Hammer *et al.* 1985), but because of the morphology and size of

Table 6.14. Collection of sheep zygotes: treatment of donors

Breed	Synchronization	Superovulation	Ovulation[a]	Insemination[a]	Embryo recovery[b]	Reference
Rambouillet	Day 10 of oestrus cycle, progesta-gen-impregnated vag. sponges; 12 days 6α-methyl-17α-acetoxy pro-gesterone, 60 mg	Twice daily 2.5 mg pFSH: starting 3 days before sponge removal until 1 day after	Onset of oestrus tested	Mated or *in utero* with 0.2 ml washed ram se-men per horn	72 hr after sponge re-moval, Ham's F_{10} + 10% FCS	Hammer *et al.* (1985)
Merino	Progestagen sponges	1,200–1,500 IU PMSG 33 hr after sponge removal	50 μg GnRH 24 hr after sponge re-moval	Intrauterine, 16 hr later (endo-scopic)	24 hr later	Nancarrow *et al.* (1987)
Welsh Mountain, Scottish Blackface, Greyface, Cheviot	12–16 days progesta-gen sponges	Two equal doses of equine 30 hr before sponge removal; FSH (1.75 or 2.15 mg)	Onset of oestrus tested	Served by at least two rams	Ovum culture medium	Simons *et al.* (1988)
Merino	14-day treatment with progesterone-impregnated intra-vag. sponges	46 hr before sponge re-moval; 12-hr inter-vals 6.4/4.2/2.2 mg FSH	24 hr after sponge removal, GnRH application	50 μl of diluted se-men into each uterine horn, 24 hr after GnRH injection	16 hr after fertil-ization, DBS + 5% sheep serum	Murray *et al.* (1989)
Merino Blackface	12–14 days, intra-vag. sponges with 40 mg fluogestone acetate	1500 IU PMSG or 17 mg FSH; 6 injec-tions every 12 hr, decreasing amounts	24 hr after sponge removal, GnRH application	Mated twice, 14–15 hr after GnRH	24–6 hr after mat-ing, PBS + 1% NBCS	Halter *et al.* (1993)

[a]GnRH, gonadotropin-releasing hormone. [b]NBCS, new-born calf serum.

Fig. 6.7. DNA microinjection into the pronucleus of an ovine zygote.

the zygotes it is very difficult to control the success of a microinjection by observing the swelling of these pronuclei.

Transfers of manipulated but noninjected zygotes showed that only 26% of the isolates developed *in vitro* to blastocysts and could be transferred to recipients. Fifty-two per cent of the transferred embryos resulted in new-born lambs. Microinjection of DNA dramatically reduced the embryonic development rate to 10% (Hammer *et al.* 1986). Rexroad and Wall (1987) found a 35% developmental rate of manipulated embryos being reduced by microinjection to 18.4%. Growing experience in handling ovine embryos and the use of more narrow injection pipettes increased the survival rate of injected zygotes to 53–71% (Walton *et al.* 1987; Murray *et al.* 1989).

Rexroad *et al.* (1990) used co-culture with ovine oviduct cells to select the embryos surviving after microinjection. The developmental rate of control embryos was 96%, that of microinjected embryos 75%. The transfer to recipients resulted in normal development of 11.3% for the control embryos and 7.3% for the microinjected embryos with pregnancy rates of 50% and 41%, respectively.

Walker *et al.* (1990) investigated the survival and developmental rate of microinjected zygotes in synthetic oviduct fluid medium. Despite a slightly lower survival rate there were no significant differences in the further development of 1- to 3-day-old embryos cultured in the synthetic medium or under *in vivo* culture conditions.

At first gene transfer experiments resulted in low embryonic survival and transgene integration rates with total efficiencies of 0.2%. Optimization of embryo manipulation and microinjection yielded, in total, efficiencies of up to 4% (Table 6.15) and integration rates of 26% (Rexroad *et al.* 1991).

6.4.2 Transgenic sheep

6.4.2.1 Gene constructs of the growth hormone cascade

At least nine different constructs containing genes of the GH cascade have been used to generate transgenic sheep (Table 6.15). So far all experiments indicate that permanent synthesis of hormones altering growth performance is not tolerated by the organism. Expression of GH or GHRF gene controlled by different promoters (mouse or ovine metallothionein I promoters, mouse albumin promoter, mouse transferrin enhancer/promoter) resulted in increased serum levels of GH and IGF-1 (Rexroad *et al.* 1989, 1990, 1991; Rexroad & Pursel 1988; Nancarrow *et al.* 1988, 1991). However, none of the transgenic sheep showed a growth performance different from that of control animals. Moreover, most transgenic animals had severe health problems such as visceromegalia (Nancarrow *et al.* 1988, 1991), diabetes (Rexroad *et al.* 1991) and pneumonia and therefore died at an early age. These results suggest that alternative strategies for expressing growth-related genes are required to modulate growth in sheep.

6.4.2.2 Wool production and metabolic pathways

Gene transfer experiments attempting to influence wool production utilize genes that increase cysteine biosynthesis in sheep (for review see Rogers 1990; Ward & Nancarrow 1991). It is well established that cysteine is a limiting factor in wool synthesis. It is impossible, however, to supply extra dietary cysteine. *Cys E* (serine transacetylase) and *cys K*/*cys M* (*O*-acetylserine sulfhydrylase) are two bacterial genes coding for enzymes that catalyse the synthesis of cysteine from sulfide and serine. Ward *et al.* (1991) successfully transferred and expressed these prokaryotic genes in mice. Rogers *et al.* (1991) introduced a gene construct consisting of both genes arranged in a tandem array into the germ-line of sheep and observed constitutive expression of both enzymes. The effects on wool production in these animals have not yet been published.

Experiments aiming to change the susceptibility of sheep to ketonuria by the establishment of novel metabolic pathways by transgenic means (for review see Ward & Nancarrow 1991) were carried out by Ward *et al.* (1991). Excess acetate in the rumen cannot be utilized for gluconeogenesis and gets converted to ketone. A biochemical pathway capable of using acetone for glucose synthesis is the glyoxylate cycle via the enzymes malate synthase and isocitrate lyase. In a model experiment, transgenic mice have been shown to express both enzymes functionally in liver, kidney and intestinal tissue. The next step will be to test these gene constructs in transgenic sheep.

Other bacterial biochemical pathways considered for establishment in transgenic farm animals include the synthesis of essential amino acids. Before these transgene experiments become a reality, a variety of technical difficulties have to be solved. The transgene expression has to be tightly controlled, so that the transferred genes do not interfere with any physiological processes. Therefore, constitutive gene expression is not practicable for most purposes. Most metabolic pathways are composed of multiple components, so that their establishment in transgenic animals requires the transfer of several gene constructs. The present state of the art does not allow the transfer of such complex gene constructs. A step towards such experiments would require greater availability of ES cells and improved techniques for handling large DNA fragments.

6.4.2.3 Gene farming

A variety of therapeutic or diagnostic proteins cannot be produced in sufficient quantity or quality by conventional methods, including prokaryotic or eukaryotic expression systems and extraction from mammalian sources. High-level expression of recombinant protein in micro-organisms has been successfully used in many cases. However, some proteins cannot be synthesized in prokaryotic expression systems due to the lack of appropriate purification procedures and more often to a deficiency of the post-translational modifications required for protein stability and/or function. Large-scale eukaryotic expression systems are technically complex, elaborate and cost intensive. As an alternative to cell culture systems, the production of large quantities of proteins in transgenic animals is appealing because of safety considerations, the high production capability of the transgenic organism, the comparatively low operating costs and the potentially un-

Table 6.15. *Gene transfer into sheep*

Construct	Embryos injected and transferred (*n*)	Offspring (incl. foetuses)		Integration		Efficiency (%)	Expression		Reference
		n	%	*n*	%		*n*	%	
mMT-I-bGH	711	38	5.3	2	5.2	0.28	2/2	100	Pursel *et al.* (1987)
oMT-oGH	436	27	6.2	1	3.7	0.23	0/1	—	Nancarrow *et al.* (1987)
mMT-I-bGH	842	47	5.6	2	4.2	0.24	2/2	100	Rexroad and Pursel (1988)
MT-hGRF	435	63	14.5	9	14.3	2.07	1/7	14	—
pMK	155	38	24.5	1	2.6	0.65	—	—	Simons *et al.* (1988)
BLG-FIX	307	57	18.6	4	7.0	1.3	2/2	100	Clark *et al.* (1989a,b)
BLG-α1AT	49	14	28.6	1	7.1	2.04	—	—	—
MisGH5	1,079	81	7.5	4	4.9	3.7	0/4	0	Murray *et al.* (1989)
MisGH 9	409	23	5.6	4	17.4	1.0	—	—	Murray *et al.* (1989)
MisGH 10	—	—	—	1	—	—	0/1	0	Murray *et al.* (1989)
BLG-AAT	439	113	25.7	5	4.5	1.1	3/3	100	Wright *et al.* (1991)
Tf-GH	247	42	17	11	26.2	4.5	3/11	27	Rexroad *et al.* (1991)
Alb-GRF	171	16	9.4	4	25.0	2.3	2/4	50	Rexroad *et al.* (1991)
cysE-cysM	803	186	23.2	33	17.7	4.1	—	—	Rogers *et al.* (1991)
pPBLac-FVIII	475	192	40.4	2	1.0	0.42	—	—	Halter *et al.* (1993)
pBBLac-FVIIIMi1	54	17	31.5	—	—	—	—	—	Halter *et al.* (1993)
pPMi-FVIII	57	21	36.8	1	4.8	1.7	—	—	Halter *et al.* (1993)
mMT-I-hGH	1,032	73	7.1	1	1.3	0.10	0/1	—	—
Total	7,701	1,048	13.6	86	8.1	1.1	15/40	38	

limited expansion of the producer animal by conventional breeding (Lovell-Badge 1985; Lathe *et al.* 1986; Mercier 1986; Church 1987; Clark *et al.* 1987; Hennighausen 1990; Wilmut *et al.* 1991; Brem *et al.* 1993).

The most appropriate organ for the expression of large amounts of foreign protein is the mammary gland because of easy access to the synthesized protein. In addition, the mammary gland has an enormous physiological potential for synthesizing proteins. The production of certain recombinant proteins might require their expression in cell types and organs other than the mammary gland because of their post-translational processing being restricted to a certain cell type. An example is the synthesis of modified antibodies in B-lymphocytes and the subsequent purification from blood of the transgenic animal (Lo *et al.* 1991; Weidle *et al.* 1991).

The feasibility of mammary-gland-specific transgene expression has been demonstrated in numerous experiments in several species (reviewed in Hennighausen 1992). Eighty per cent of farm animals' milk consists of six main proteins (four caseins, β-lactoglobulin and α-albumin) being secreted from epithelial cells of the mammary gland under the control of several hormones (Clark *et al.* 1992).

The main milk protein of sheep is β-lactoglobulin (BLG) with an average concentration of 3–5 g/l. The BLG mRNA represents approximately 5% of total mRNA of mammary gland cells (Clark *et al.* 1989a). Ovine BLG regulatory sequences promote tissue-specific high-level transgene expression in mouse models. The transgene expression with BLG promoter constructs was tightly controlled and resembled the endogenous mouse β-casein gene expression profile (Simons *et al.* 1987; Harris *et al.* 1991).

Human α_1-antitrypsin (hα_1AT) is a glycoprotein which is normally present at 2 g/l in plasma. Genetic deficiencies in circulating concentrations of hα_1AT are a common lethal hereditary disorder affecting males and leading to life-threatening emphysema. The annual demand for replacement therapy using human-plasma-derived α_1AT is more than 4,000 kg. Therefore, recombinant-DNA-derived sources are highly desirable. Archibald *et al.* (1990) reported the production of biologically active hα_1AT at yields of up to 7 mg/ml in the milk of transgenic mice expressing a gene construct containing the sheep BLG promoter fused to hα_1AT coding sequences.

To form the basis for a manufacturing process by transgenic means, a BLG–hα_1AT gene construct was used to generate five transgenic

sheep (four female, one male) (Simons *et al.* 1988; Wright *et al.* 1991). Three of the transgenic females expressed the human protein, all at levels greater than 1 g/l. In one case, initial levels exceeded 60 g/l and stabilized at 35 g/l during the lactation period. hα_1TA purified from sheep milk was fully glycosylated and exhibited biological activity indistinguishable from human-plasma-derived protein. In the high-level expressing animal, the recombinant protein represented nearly 50% of total milk protein, considerably exceeding the production levels of α_1AT obtained in other expression systems.

In a second experiment Simons *et al.* (1988) generated five transgenic sheep harbouring gene constructs consisting of the ovine BLG promoter fused to the human blood coagulating factor IX. Two female transgenic animals secreted the foreign protein into their milk. However, the transgene transcription was approximately 1,000-fold lower than endogenous BLG expression (Clark *et al.* 1989b).

6.5 Gene transfer into goats

The reproductive and milk production data for goats are very similar to those for sheep. Therefore, it is surprising that the first gene transfer experiments in the goat have been initiated only recently (Ebert *et al.* 1991; Ebert & Schindler 1993). This is probably because in most countries investigating gene transfer into farm animals, goat production is traditionally not of great importance.

6.5.1 Treatment of donors and recipients

Several protocols are currently used to isolate oocytes from goats. Since goats are seasonally anoestrus, the efficiency of collecting and transferring embryos varies with season. The oestrus of donors is synchronized by administration of Norgestomet ear implants (Pendleton *et al.* 1986; Selgrath *et al.* 1990; Ebert *et al.* 1991). Alternatively, progesterone-containing vaginal sponges or progesterone injection is used for donor synchronization (Armstrong *et al.* 1987; Nuti *et al.* 1987; Cameron *et al.* 1988). The progesterone treatment is carried out for 11–18 days. Superovulation is induced by either PMSG or FSH, ideally 10–48 hr after the progestagen treatment has ended. The induction scheme follows mainly that of sheep (see Table 6.14). Luteolysis can be increased by treatment with progesterone $F_{2\alpha}$ 36 hr before induction of superovulation. Further optimization of synchronization

Table 6.16. *Embryo collection and transfer in a transgenic goat programme*

	n	$\bar{x}$	%
Donors	269	—	—
Ovulations	2,356	8.8	—
Recovered	1,429	5.3	60.6
Fertilized	1,058	3.9	74.4 (44.9)[a]
Recipients	190		
Embryos transferred	782	4.1[b]	
Pregnant recipients	107		56[c]
Live young	169	1.6	22[d]
Transgenic kids	12	1.6	7.1[e]

[a] Percentage of embryos collected per ovulation.
[b] Embryos per recipient.
[c] Pregnancy rate (oviduct and uterus transfer).
[d] Survival rate of embryos transferred.
[e] Integration rate (transgenic per live young).
Source: Ebert and Schindler (1993).

and ovulation can be achieved by administration of gonadotropin-releasing hormone (GnRH) 24–6 hr after the prostaglandin treatment is ended. When signs of oestrus are observed, the donor animals are mated several times to fertile males or inseminated artificially over a 2-day period.

Embryos are recovered surgically 48–72 hr after the progesterone treatment is finished. On average, superovulation yielded 7.7–19.4 zygotes per goat (Armstrong *et al.* 1987; Fabricant *et al.* 1987; Selgrath *et al.* 1990). The best results were obtained using FSH.

The pronuclei of fertilized oocytes are visualized either under Nomarski's interference-contrast microscopy or after centrifugation (7,000 *g*, 2 min). Microscopy is sometimes difficult owing to large lipoid granula in the cytoplasm.

The preparation of recipients is similar to that of donor animals. Embryos are transferred surgically into one oviduct of synchronized recipients. Table 6.16 summarises the results of the embryo transfer programme of Ebert and Schindler (1993).

Embryo and gene transfer experiments in goats resulted in overall pregnancy rates of 56% and a total gene transfer efficiency (transgenic goats per microinjected embryos transferred) of 1.5% (Table 6.17).

Table 6.17. *Gene transfer into goats*

Construct	Zygotes and embryos injected and transferred (*n*)	Offspring (incl. foetuses)		Integration		Efficiency (%)	Expression		Reference
		n	%	*n*	%		*n*	%	
mMT1-oGH	153	9	5.9	0	0.0	—	—	—	Fabricant *et al.* (1987)
WAP-LAtPA	203	29	14	2	6.9	1.0	1/2	50	Ebert *et al.* (1991)

6.5.2 *Transgenic goats*

So far only a few gene transfer experiments involving goats have been carried out. The experiments of Fabricant *et al.* (1987) using GH gene constructs failed to generate transgenic goats. The first successful production of transgenic goats expressing a heterologous protein in their milk was reported by Ebert *et al.* (1991). The goat was chosen to establish a commercial prototype for the large-scale manufacture of high-value proteins in the transgenic mammary gland for several reasons: dairy goats produce large quantities of milk (on average 4 l/day); goats have gestation and development periods of moderate length (5 and 8 months, respectively); and the biochemistry of goat milk has been well described.

Tissue plasminogen activator (tPA) plays a key regulatory role in the orderly progression of wound-healing processes and has a potential clinical value in the treatment of coronary thromboses. A glycosylation variant of human tissue plasminogen activator (longer-acting tPA or LAtPA), controlled by the murine WAP promoter was expressed in the mammary gland of a transgenic dairy goat. Milk was obtained upon parturition of the goat and contained enzymatically active LAtPA at a concentration of 3 μg/ml. On average the transgenic goat produced 10–15 mg foreign protein per day during the lactation period. Denman *et al.* (1991) developed a purification protocol for LAtPA from the milk of the transgenic goat by combining acid fractionation, hydrophobic interaction chromatography and immunoaffinity chromatography. The procedure resulted in 8,700-fold purification with a cumulative recovery of 25%. The specific activity of the purified transgenic protein was 6.1×10^5 U/mg. This was approximately 84% of the value observed for the recombinant protein synthesized in an *in vitro* expression system based on mouse cells. Although the transgenic protein was biologically active, there were significant differences in the oligosaccharide structures between *in vitro* expression-system-derived and transgenic LAtPA. As with any recombinant protein synthesized in different hosts, the consequences of structural differences on therapeutic utility require evaluation on a case-by-case basis. Another gene construct consisting of LAtPA regulated by a β-casein promoter was used to produce a transgenic goat expressing the protein at 2–3 mg/ml (Ebert & Schindler 1993). Unfortunately, the lactation of this goat stopped for unknown reasons.

6.6 Gene transfer into cattle

Although cattle are the most important species in agricultural production, they have rarely been used for gene transfer experiments. This is due to the nature of their reproduction (i.e., a long generation interval of 2–3 years with normally only one offspring per gestation), to difficulties in collecting and transferring early embryo stages and to the generally high cost of cattle husbandry. The development of procedures for *in vitro* production and *in vitro* culture of bovine embryos has dramatically reduced the technical problems.

6.6.1 *Treatment of donors and recipients*

Preparation of donor and recipient animals in gene transfer programmes is carried out according to procedures developed in commercial embryo transfer programmes. If necessary the oestrus cycles of donors and recipients are synchronised by $PGF_{2\alpha}$ administration. The gonadotropic substances used for the induction of superovulation include PMSG, FSH or a combination of FSH and luteinizing hormone. PMSG has the advantage of a long half-life (40–120 hr) and hence need be administered only once between days 11 and 14 after the onset of oestrus (dosage of 2,000–3,000 IU). Ovulation is induced by administration of prostaglandin 2 days after the PMSG injection. After 48–64 hr, the donors are inseminated. The most convenient time for embryo collection is 75–96 hr after the prostaglandin treatment.

Embryo isolation from oviducts is usually achieved by one of several procedures: upon slaughtering (Biery *et al.* 1988), surgical flushing of the oviducts (Loskutoff *et al.* 1986; McEvoy & Greenan 1990) or removal of the ovary and oviduct by castration (Roschlau *et al.* 1989). The collection of embryos upon slaughtering is usually the method of choice, since the other procedures are too laborious and cost intensive. On average, 12 oocytes can be collected per donor animal, of which half are suitable for microinjection.

The pronuclei of bovine embryos have to be visualized by centrifugation. Controlling the microinjection is frequently difficult because the pronuclei are often blurred even after centrifugation. Lohse *et al.* (1985) injected a thymidine kinase construct into bovine embryos to estimate the success rate of the microinjection. Approximately 30% of the injected embryos showed transient expression of thymidine kinase activity and therefore demonstrated successful microinjection.

The effects of manipulation of bovine embryos (i.e., centrifugation, microinjection and culturing) on their survival rates and further development have been investigated by several laboratories (McEvoy *et al.* 1987; Hawk *et al.* 1989; Peura *et al.* 1993). They found that the gene injection alone seemed responsible for decreased embryonic development, whereas treatments required for microinjection (centrifugation and microscopic evaluation) and *in vivo* culture (e.g., in rabbit oviducts) did not have detrimental effects. Reichenbach *et al.* (1991) have tested various *in vivo* culture systems (temporary culture in oviducts) as well as *in vitro* cultures (see Section 6.6.2; Table 6.18). On average 20% of the microinjected embryos showed further development after *in vivo* culture in bovine, porcine or rabbit oviducts and subsequent transfer into recipients. *In vitro* culture with bovine epithelial cells resulted in a developmental rate of 15%. Jura *et al.* (1992) achieved in separate experiments with *in vivo* and *in vitro* culture systems 11 and 6 pregnancies by transferring 63 and 39 embryos, respectively.

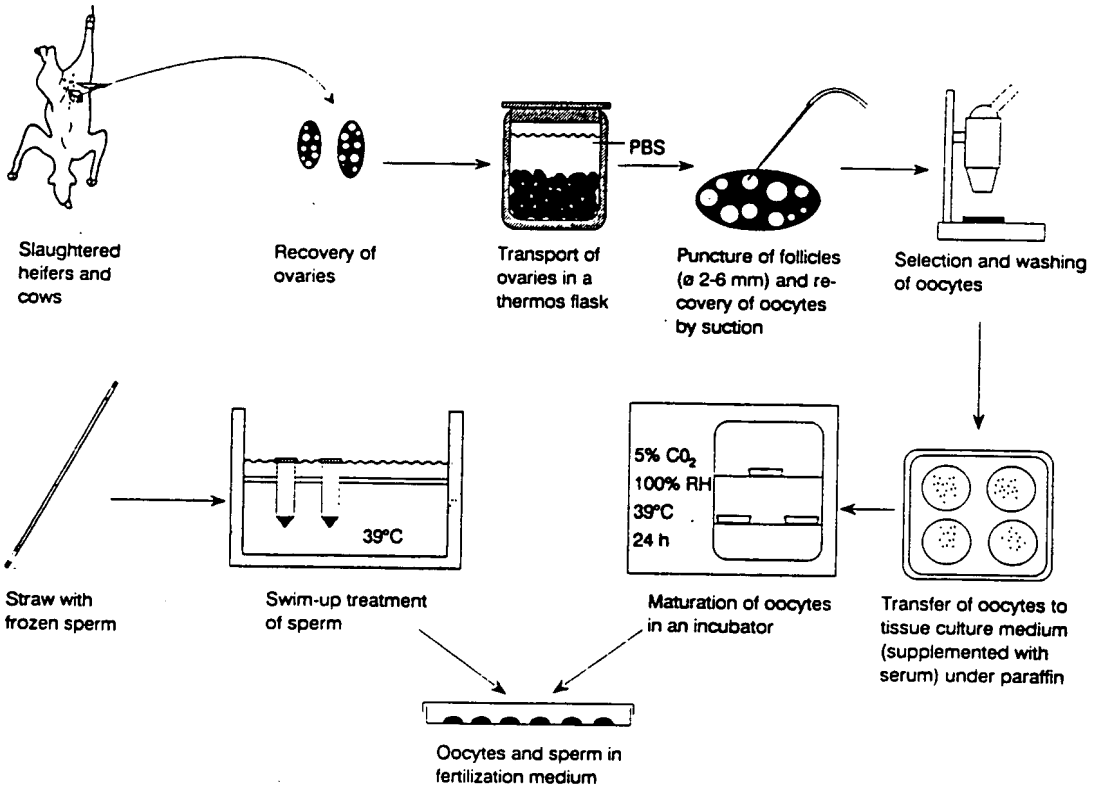
6.6.2 In vitro *production of bovine zygotes*

A scheme for the *in vitro* production of zygotes suitable for microinjection from ovaries of slaughtered cows has been developed in our laboratory and is depicted in Fig. 6.8. A similar procedure has been described by other laboratories as well. On average 15 intact cumulus–oocyte complexes per ovary were collected and subsequently matured in modified tissue culture medium 199 (TCM 199) (Pavlok *et al.* 1988) supplemented with heat-inactivated serum of cows in oestrus. The *in vitro* fertilization was performed with cryo-conserved sperm (10^6 sperm cells per millilitre) (Parrish *et al.* 1986) in fertilization medium (Ball *et al.* 1983). The fertilized oocytes were co-cultured with cumulus-derived granulosa cells in modified TCM 199 for a further 90 hr (Berg & Brem 1989). Thirty-two per cent of the oocytes matured and fertilized *in vitro* (fertilization rate of 68%) showed further development to the morulae/blastocysts and therefore reached the developmental stage, providing the possibility of nonsurgical transfer into recipients (Reichenbach *et al.* 1992). In total, 74% of the cultured embryos overcame the developmental block at the 8- to 16-cell stage. Initial experiments used 311 *in vitro* matured and fertilized oocytes for microinjection (Fig. 6.9). Further development was shown by 45 injected zygotes, which were transferred nonsurgically into 29 recipients. This resulted in 9 pregnancies (pregnancy rate of 31%) (G. Brem

Table 6.18. In vivo *and* in vitro *development of microinjected bovine zygotes*

Culture system	Recipients		Zygotes and 2-cell embryos transferred (D1–D7)	Embryos recovered (D5–D1)		Embryos developing ≥1 cell division		Embryos developing morulae and blastocysts	
	n	type	*n* (a)	*n* (b)	% (b/a)	*n* (c)	% (c/b)	*n* (d)	% (d/b)
In vivo									
Bovine	8	MI	183	93	51	24	26	17	18
	3	C	66	48	73	27	56	14	29
Rabbit	4	MI	82	46	56	28	61	13	28
	2	C	41	31	76	21	68	14	45
Swine	3	MI	79	22	28	7	32	3	14
	1	C	29	12	41	5	42	2	17
Total	21	MI/C	480	252	53	112	44	63	25
In vitro	—	MI	123	123	100	63	51	18	15
	—	C	71	71	100	43	61	21	30

Abbreviations: D, day; MI, microinjected embryos; C, control group (not microinjected).
Source: Reichenbach *et al.* (1991).



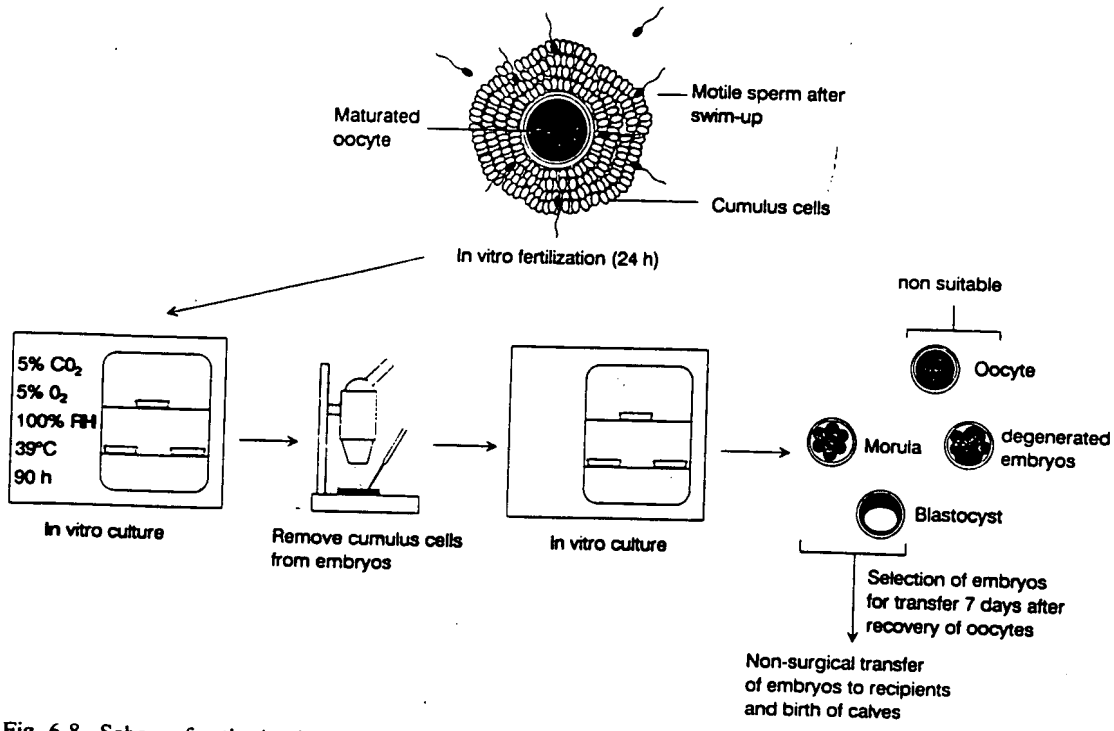


Fig. 6.8. Scheme for the *in vitro* production of bovine zygotes.

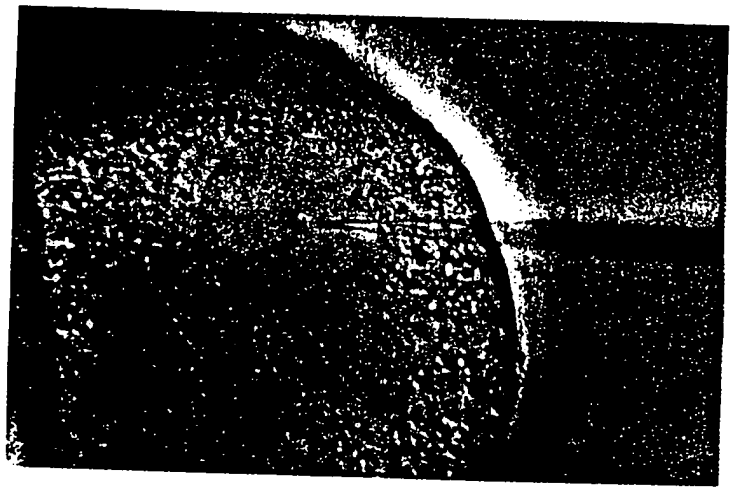


Fig. 6.9. DNA microinjection into the pronucleus of a bovine zygote.

et al. unpublished data). Meanwhile, similar results (Table 6.19) have been reported by Massey (1990), Krimpenfort *et al.* (1991) and Hill *et al.* (1992). *In vitro* production of bovine embryos provides a cheap and simple source of oocytes and early embryonic stages for gene transfer experiments. In addition, embryos produced *in vitro* show less variability in the time schedule of their developmental stages than embryos collected from superovulated donors and are therefore notably easier to manipulate successfully.

6.6.3 *Transgenic cattle*

So far only a few experiments resulting in the generation of transgenic cattle have been published. As yet there are no data available concerning the expression of transferred genes in these animals.

In an early experiment Biery *et al.* (1988) injected the bacterial chloramphenicol transferase gene controlled by the Rous sarcoma virus promoter (RSV-CAT) into the pronuclei of embryos and subsequently cultured them *in vivo* in ovine oviducts. Twenty-one per cent of the injected embryos developed to the morula/blastocyst stage and were nonsurgically transferred into synchronized recipients. The analysis of 79 foetuses on day 60 of the pregnancy resulted in 4 transgenic foetuses.

Roschlau *et al.* (1989) reported the generation of a transgenic calf by

Table 6.19. *Gene transfer into cattle*

Construct	Zygotes and embryos injected and transferred (*n*)	Offspring (incl. foetuses)		Integration		Efficiency (%)	Reference
		n	%	*n*	%		
α-Fetoprotein	852	—	—	4	—	0.5	Church *et al.* (1986)
RSV-CAT	819	79	9.6	4	5.1	0.49	Biery *et al.* (1988)
pMMTV-bGH	201	14	7.0	1	7.1	0.5	Roschlau *et al.* (1989)
ASK-HER	1,704	79	4.6	1	6.3	0.3	Massey (1990)
α_{s1} *cas-hLF*	1,154	21	1.8	2	9.5	0.2	Krimpenfort *et al.* (1991)
ASKHER	4,150	53	1.5	1	1.9	0.02	Hill *et al.* (1992)
202 aASK-IGF-I	7,810	67	0.9	3	4.5	0.04	Hill *et al.* (1992)
MMTV-IGF-I	1,336	20	1.5	2	10	0.01	Hill *et al.* (1992)
733 ASK-GF-I	6,070	53	0.9	1	1.9	0.02	Hill *et al.* (1992)
Total	24,096	386	1.7	19	3.9	0.08	

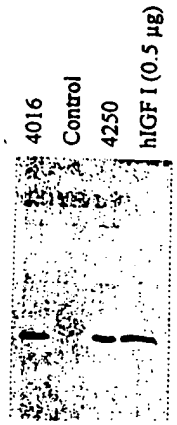


Fig. 6.10. Western blot analysis of IGF-1 expression into the milk of transgenic rabbits.

injecting an MMTV–*bGH* gene construct into zygotes collected from superovulated donors by ovariohysterectomy.

Hill *et al.* (1992) summarize the results of a gene transfer programme using four different gene constructs (three of them containing the gene coding for IGF-1). A total of 14.5% of the injected embryos developed to morulae and blastocysts and were transferred into recipients (pregnancy rate of 31%). Ninety-nine new-born calves were analysed, 3 of which harboured copies of the injected gene construct.

Bowen *et al.* (1993) reported the generation of transgenic cattle from PCR-screened embryos. Fertilized oocytes were microinjected with different gene constructs (chicken *c-ski* gene; bovine leukaemia virus envelop glycoprotein gene) and subsequently cultured *in vivo* in rabbit oviducts for 5–6 days. Recovered embryos were microsurgically biopsied, and parts of the trophoblast subjected to PCR. Transgenic embryos were transferred nonsurgically to recipients for further development. Transfer of 112 PCR-positive embryos resulted in 28 foetuses, of which 2 were transgenic. Despite the problem of false positives caused by short-term persistence of small quantities of the microinjected DNA in the embryo, the system of assessing transgenic status in embryos before transfer into recipients reduced the number of recipients needed by 79%.

Our own experiments concentrate on the generation of transgenic cattle with mammary-gland-specific expression of foreign proteins.

Assuming a daily milk production of 30 litres containing 35 g of protein per litre, cattle synthesize at least 1 kg of milk protein per day. It has been estimated that the proportion of recombinant proteins in milk can be 10%, but there is a subsequent 30% loss during protein isolation, leading to an annual production capacity of 15 kg of highly purified recombinant protein per cow. Denman *et al.* (1991) have demonstrated the feasibility of large-scale purification of foreign protein from the milk of transgenic animals. The bovine α_{s1}-casein promoter was chosen to direct the expression of the desired protein to the mammary gland. In a pilot project, transgene expression controlled by the α_{s1}-casein promoter was tested in transgenic rabbits. Transgenic rabbit lines were established in which the females expressed on average 0.5–2 g/l foreign proteins (Fig. 6.10) during their lactation (Brem & Hartl 1991; for review see Brem *et al.* 1993). Transgene expression in all transgenics was exclusively found in the mammary gland. These experiments clearly demonstrated the feasibility of the bovine α_{s1}-casein expression cassette to direct high-level expression of foreign proteins to the mammary gland of transgenic animals.

Krimpenfort *et al.* (1991) used a gene construct containing the coding region of the human lactoferrin gene *(hLF)* controlled by α_{s1}-casein regulatory sequences in a gene transfer programme. The microinjected embryos were produced *in vitro;* 2 of the 19 new-born calves were transgenic.

The success in producing bovine embryos *in vitro,* generating transgenic dairy cattle and expressing foreign proteins in transgenic animals using a bovine mammary-gland-specific transcription cassette has resulted in significant progress towards the future utilization of transgenic cattle in gene farming.

6.7 Breeding with transgenic animals

The basis for the establishment of transgenic lines is the integration of the microinjected gene construct into the germ-line of the primary transgenic animal. Even though DNA is injected directly into the pronuclei of fertilized oocytes, there is the possibility of generating animals which contain cells of different genotypes. These so-called transgenic mosaics might be inappropriate for establishing transgenic lines if the founder animal has no germ-line integration of

the gene construct. Approximately 30% of all primary transgenic animals generated by microinjection are mosaics (Wilkie *et al.* 1986). Normally, mosaicism occurs only in primary transgenic animals, because mating results in transgenic progeny harbouring the transgene in all cells.

The transgene usually follows Mendelian inheritance if it has been integrated at a single chromosomal locus. Such animals are defined as hemizygous. The description 'heterozygous' is inapt since the corresponding chromosome lacks the transgenic allele. Mating of hemizygous transgenic animals theoretically results in 25% homozygous and 50% hemizygous transgenics, as well as 25% nontransgenic animals. In most transgenic lines the transgene is transmitted stably over several generations.

Occasionally, a primary transgenic animal shows more than one chromosomal integration site of the transgene. The Mendelian inheritance pattern in an animal with two independent integration sites results in 25% nontransgenic and 75% transgenic progeny (25% harbouring both loci and 50% inheriting only a single locus).

The random transgene integration may cause an insertional mutation by disrupting an endogenous gene, the effects being predominantly observable in homozygous animals. If the transgene has inserted in a vital endogenous gene, the subsequent mating of hemizygous animals will never yield homozygous progeny.

Producing transgenic progeny by conventional breeding with primary transgenic animals has been described for pigs (Pursel *et al.* 1987; Brem *et al.* 1990), sheep (Rexroad *et al.* 1988; Nancarrow *et al.* 1991) and goats (Ebert *et al.* 1991). Our own investigations of 16 transgenic founder pigs carrying four different gene constructs are summarized in Table 6.20. Approximately 50% of the primary transgenic pigs were mosaics, resulting in less transgenic progeny than expected. Mating of transgenic F1 animals resulted in homozygous transgenic piglets (Table 6.21). No signs of deleterious insertion mutations have been observed. These results obtained by mating transgenic farm animals demonstrated the establishment of transgenic lines with stable integration of the gene constructs.

6.8 Conclusion

Methods of gene transfer in farm animals are rather new and still very expensive. In the future, technical optimization should lead to substantial reductions in cost. In addition, the further elucidation of the genetics of production traits – i.e., characterization of 'major genes' – will make it possible to alter the phenotype of domestic animals by designing appropriate gene constructs.

The application of gene transfer to farm animals depends on the acceptance of this technology by the public as well as the introduction of appropriate laws permitting the use of transgenic animals in the production of food and pharmaceuticals or as sources of organs for transplantation.

Table 6.20. *Transmission of transgenes to F1 offspring of transgenic founder pigs*

Sex	Litters (*n*)	Progeny (*n*)	Transgenic (*n*)	Per cent
M	3	32	17	53
M	15	132	10	8
M	4	33	17	52
M	5	42	18	43
M	9	104	15	14
M	6	53	26	49
M	9	92	22	27
M	4	28	18	64
$\bar{x}$	55	516 (9.4)[a]	143	28
F	1	12	7	58
F	3	27	—	—
F	2	13	—	—
F	1	7	4	57
F	1	7	5	72
F	1	9	2	22
F	2	11	3	27
F	3	31	7	23
$\bar{x}$	14	117 (8.4)[a]	28	24
Total	69	633 (9.2)[a]	171	27

[a] Number in parentheses is average of litter size.

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Table 6.21. *Breeding of homozygous transgenic (TG) pigs*

	Mating of transgenic × transgenic						
TG pig line	Litter (*n*)	Piglets (*n*)	TG (*n*)	Non-TG (*n*)	TG/non-TG (%)	Homozygous TG (*n*)	TG/homozygous TG (%)
2980	13	112	33	12	73	10	30
2977	8	73	26	11	70	8	22
2973	3	22	16	6	73	8	36
2380	18	186	8	3	73	3	27
4243	1	10	7	3	70	1	10
Total	44	403	90	35	72	30	24

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7

Minor transgenic systems

NORMAN MACLEAN

7.1 The range of transgenic animals

As seen in previous chapters, animal species of many kinds have been used for experiments in transgenic induction. Some offer particular benefits to the experimentalist; others have proved difficult or even impossible to use. In this book we have attempted to review all of those in which transgenic induction has been successful. The present range is as follows: protozoans such as *Paramecium* and *Trypanosomid* species, a few species of sea urchins, the nematode *Caenorhabditis elegans*, a few insects such as the silk moth *Bombyx*, the mosquito *Anopheles* and the fruit fly *Drosophila*, the predatory mite *Metaseiulus*, fish of many species, the amphibian *Xenopus laevis*, the domestic chicken and both laboratory mammals such as rats, mice and rabbits and large mammals such as cattle, sheep and pigs.

The failures and successes of applying transgenic technology to all of these experimental systems makes fascinating reading, yet clearly we are only at the threshold of this interesting study area. As well as the major transgenic systems that we have addressed here, there are a number of interesting minor ones, which will be briefly reviewed in this chapter. Of course, since this book is about transgenic animals, we have not discussed the extensive work on transgenic plants, fungi such as yeast and *Aspergillus* or bacteria, in which transgenism is a way of life, facilitated as it is by the extensive range of natural plasmids.

7.2 Protozoans

It is relatively easy to produce transgenic forms in at least some groups and species of Protozoa. Thus, following the work of Cruz

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Contents

SECRETION OF HUMAN FURIN INTO MOUSE MILK

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Abstract

We demonstrate here the synthesis of the human proprotein processing enzyme, furin, or paired basic amino acid-cleaving enzyme (PACE) in the mouse mammary gland. Bigenic mice were generated for two transgenes, the mouse whey acidic protein (WAP) promoter/ PACE cDNA and WAP/ human Protein C (HPC) gene, to improve the endoproteolytic processing of the recombinant HPC (rHPC) precursor. Both rHPC and furin transgenes were expressed in the same regions of the mammary gland, and regulated in milk-filled and empty alveoli similar to the endogenous milk protein, WAP. In addition to localization in the Golgi of epithelial cells, furin was detected in milk-filled lumina. Furin in milk was a truncated form of the intracellular enzyme, lacking the transmembrane and cytoplasmic domains. The secreted enzyme was active and processed the rHPC precursor in milk. Thus, we have not only shown that the proteolytic processing capacity of specific organs can be enhanced, but that secreted forms of human protein processing enzymes may be produced and protein processing continued in the milk of transgenic animal bioreactors.

Several researchers have employed transgenic animal bioreactors (TABs) for the production of therapeutic human proteins. Expression of foreign genes has been targeted to selected tissues using specific promoters and the protein collected from bodily fluids like milk, blood, urine or saliva (1). Detailed analysis of some proteins produced in TABs have identified differences in posttranslational modifications, as compared to their human counterparts (2, 3, 4). These may affect their biologic and pharmacologic properties. While modern methods of purification allow for the selection of active subpopulations or those with different glycoforms of proteins (5), the remodelling or engineering of animal organs is another approach. We have developed the next generation of TABs by raising the levels of a posttranslational protein processing enzyme in the mammary gland by co-expression of the proprotein processing enzyme furin (6), or paired basic amino acid cleaving enzyme (PACE) with human Protein C (HPC), resulting in enhanced proteolytic maturation of the rHPC precursor in the mammary gland (7). Here we demonstrate the localization of Golgi protease furin in the mammary gland of transgenic mice and its secretion into the milk. The secreted enzyme is active, cleaving both peptide and protein substrates. Thus, limiting factors in the synthesis of heterologous proteins, such as levels of endogenous processing enzymes may be overcome, and active forms of enzyme may be secreted, with protein processing continuing in milk.

Results and Discussion

Detection of furin in the mammary gland. Histologic analyses were performed to determine furin expression and subcellular localization in the mammary glands of HPC/PACE transgenic mice (7). The gross morphology and histology of mammary glands from transgenic mice at mid-lactation was similar to that of control. Tissues were probed with antibodies to rHPC and furin,

and the localization of synthesized protein was compared to that of endogenous whey acidic protein (WAP). Immunostaining of serial sections demonstrated that mammary alveoli synthesizing rHPC also contained furin. Thus both transgenes were expressed in the same region of the mammary gland, in the same alveoli, suggesting coordinated regulation of two transgenes in the mammary gland. The presence of WAP in these alveoli showed that the expression of two transgenes did not prevent the expression of endogenous milk protein genes, and that the cellular pattern of expression of the 4.1 kb WAP promoter-driven transgenes was similar to that of endogenous WAP.

Unlike WAP and rHPC, furin showed a more discrete intracellular staining pattern, suggesting retention in the trans-golgi network or in post-Golgi secretory vesicles of the alveolar epithelial cells. Furin was also detected in the alveolar lumen, indicating partial secretion of this intracellular protein. This was unexpected, as furin is predominantly a Golgi membrane-localized protein (8) and naturally secreted forms have not been identified in any bodily fluid. Both furin and rHPC were detected mainly inside the epithelial cells of milked-out alveoli, whereas in milk-filled alveoli, staining was more intense in the lumina. Thus, it appears that cellular synthesis of the processing enzyme regulated by milk gene sequences is decreased when alveoli are fully distended with milk and occurs primarily after the alveoli discharge their contents, similar to the regulation of endogenous WAP synthesis.

Secretion of furin into milk. To confirm the histological observations, we analyzed the milk of HPC/PACE transgenic mice for the presence of secreted furin. The furin-specific MON 148 monoclonal antibody which recognizes an epitope in the catalytic domain (9) was used to probe western blots of milk proteins from control and transgenic mice. A band of approximately 80

KDa was detected in the milk of mice from all four transgenic lines (Fig. 2A), confirming secretion of furin into milk. Western blotting with antibodies recognizing different epitopes led to the detection of predominantly the 80 KDa protein by MON 148 and 152 (Fig. 2B). Human-furin-specific MON 152 detected a faint band at about 90 KDa in line C5.2 milk, but this result was not consistent, nor observed in milk from other lines (data not shown). The faint bands detected by MON 148 between 116 and 205 KDa might be due to complexation of furin with milk proteins. Probing with MON 150 which recognizes an epitope in the prepro-region indicated that the propeptide of furin had been removed, while lack of detection with MON 139 suggested absence of the C-terminal transmembrane/cytoplasmic region. The proteins detected in both control and transgenic milk at 50 and 100 KDa were mouse IgG (H) chain monomers and dimers, respectively, recognized by the secondary antibodies.

These results are consistent with truncation at and removal of the hydrophobic transmembrane domain of furin, resulting in a decrease in molecular weight from 90/94 KDa to 80 KDa. A minor band around 65 KDa was also observed and is probably due to additional cleavage in the Cysteine-rich region of furin. Thus, specific posttranslational processing of the C-terminus of furin occurs in mammary epithelial cells and/or in milk. Upon overexpression of furin, a 75 KDa truncated form was secreted from COS-1 (10) cells and an 80 KDa form from BSC40 cells (11), but this is the first report of secretion in animals.

Activity of secreted furin. We have previously shown that rHPC secreted into the milk of HPC transgenic mice consisted of 40%-60% single chain precursor form, with 20-30% containing the attached propeptide (2). This was due to inefficient intracellular cleavage of the Lys-Arg dipeptide linking the light and heavy chains at -Arg-Ser-His-Leu-Lys-Arg^{157}-↓-, as well

as of the propeptide at -Arg-Ile-Arg-Lys-Arg^{-1}-↓-. However, rHPC secreted into the milk of HPC/PACE bigenic mice was well processed (7). To determine if secreted furin could process the rHPC precursor in milk, milk from HPC/PACE mice was added to HPC milk in a 10:1 ratio and incubated for 0-30 min, at 37°C. This resulted in considerable reduction of the amount of rHPC single chain, as monitored by western blotting with two antibodies directed against HPC (Fig. 3A). Incubation of HPC milk alone did not result in processing, while the trace amounts of single chain present in HPC/PACE milk disappeared. This shows that furin secreted into milk has biological activity, in contrast to the PACE-Sol mutant secreted into the medium of COS-1 cells (12). PACE-Sol secreted by CHO cells did cleave recombinant factor IX when cocultured, although at a much slower rate than when coexpressed (13). An unidentified mouse protein migrating faster than the rHPC single chain was also detected by the secondary goat anti-mouse IgG antibody (lanes 1-6).

To ascertain whether secreted furin could also remove the propeptide from pro-rHPC secreted into milk, a mixture of milk from HPC and HPC/PACE mice was incubated for 3 hrs, at 37°C. rHPC purified from this mixture was compared by SDS-PAGE to rHPC from the milk of HPC and HPC/PACE bigenic mice. Results show a dramatic decrease in the amount of single chain rHPC precursor in the mixture (Fig. 3B). The purified protein was subjected to automated Edman degradation and the amino acid sequence compared to that of plasma-derived HPC (Fig. 3C). These data show conclusively the site-specific cleavage of the propeptide from rHPC by secreted furin.

The specific activity of furin was determined by cleavage of a fluorogenic tetrapeptide substrate, boc-RVRR-AMC. Furin in milk of all lines cleaved the substrate, releasing AMC. The

specific activity of the enzyme varied according to the level of furin present in milk, Table I and Fig. 2A. The K_m for cleavage of boc-RVRR-AMC by furin was determined to be about 32 μM and correlates well with the value of 27 μM reported for furin from VV:hFUR (11) and 26 μM for hFUR713t (14) infected BSC40 cells.

Implications for the production of processed proteins and processing enzymes. The possible systemic effects of furin expression on animal health and mammary function could not be excluded. Toxicity of furin upon high level expression in transfected cells has been reported (15) and is probably due to promiscuous processing of other protein substrates. Females from two of the four lines lactated normally, probably because the mammary gland is an actively secreting organ which secretes the excess enzyme into milk. Another possibility is that the presence of sufficient amounts of a specific substrate, namely rHPC, prevents access of furin to other proteins. We believe that impaired lactation in the other two lines is primarily due to differences in the developmental regulation of transgene expression (Drews *et al.*, unpublished observations).

Our results should encourage others to augment the processing of heterologous proteins in TABs by coexpressing essential enzymes. It will not be necessary to perform *in vitro* processing of poorly modified proteins after synthesis in TABs, instead this can be done *in vivo*. The presence of furin inside the cell, as well as, in milk allows for a longer interaction with its substrate. Thus, coordinate localization and the "residence time" of rHPC in the Golgi need not be limiting factors in precursor processing by furin (Fig. 4.) Reduction of temporal and spatial constraints on protein processing highlight further advantages of protein expression in the mammary gland bioreactor system. We have described a method to synthesize up to 41,000

U/ml of an active, secreted form of a posttranslational processing enzyme which is normally intracellular and membrane-bound, without toxicity to animals. In the future, this will allow us to purify such enzymes from milk for further structure-function studies, as well as to design possible inhibitors and drugs to modulate their action.

Experimental Protocol

DNA constructs and generation of transgenic animals. The WAP/PACE construct comprising the 2.47 kb human furin cDNA and 74 bases of 3' untranslated sequence, as well as, the generation of transgenic mice by coinjection of the WAP/HPC (2) and WAP/PACE DNA fragments, Fig. 4, into fertilized embryos of CD-1 mice (Charles Rivers) has been described (7).

Immunohistochemistry. Paraffin-embedded serial sections of mammary gland from mid-lactation (day 10) WAP/HPC transgenic mice were de-paraffinized and stained with hematoxylin/eosin, or immunostained. Tissues of at least two animals from lines C1.2, C2.2 and C5.2 were analyzed. Overall, the histological appearance of tissues from each line were similar. Endogenous peroxidase activity was exhausted by immersion in 0.3% hydrogen peroxide for 30 min. Sections were preincubated in 1% BSA/PBS with 10% rabbit, horse or goat serum for the detection of rHPC, furin and mWAP, respectively. rHPC was detected with a 1:400 dilution of sheep anti-HPC antibody (Affinity Biologicals), furin with a 1:5 dilution of MON 148 antibody (gift of Dr. Van de Ven; 9) and mWAP with a 1:800 dilution of rabbit anti-mWAP antibody (gift of Dr. Hennighausen). The respective biotinylated secondary antibodies at 1:1000 to 1:2000 dilutions, ABC Elite kit (Vector Labs.) and DAB substrate (Sigma) were used to develop the sections, which were then counterstained with Mayer's hematoxylin.

Western blot detection of furin in milk. Continuously bred mice were milked between days

7 and 15 of lactation after i.p. administration of 0.3 ml (0.5 IU) oxytocin (Sigma). Milk was diluted with 2 volumes of phosphate-buffered saline (PBS), pH 7.4 containing 50 mM EDTA, centrifuged twice at 15,000 rpm for 15 min at 4^0 C and stored at -80^0 C. Milk proteins and prestained standards (BioRad) were separated under reducing conditions on 8-16% (Novex) or 10% SDS-polyacrylamide gels and silver stained, or transferred to nitrocellulose membranes. Blots were serially reacted with either the MON 139, 148, 150 or 152 monoclonal antibodies recognizing different epitopes of furin and HRP-conjugated secondary antibodies, then developed by enhanced chemiluminescence (Amersham). rHPC was detected using the anti-HPC 8861 monoclonal antibody, recognizing a heavy chain epitope, or a sheep polyclonal antibody (Affinity Biologicals).

Activity of furin. Fluorometric assays were carried out essentially as described (14). Defatted milk diluted 1:100 to 1:400 was incubated with an equal volume of 100 μM peptide substrate boc-RVRR-AMC (N-α-t-butoxycarbonyl-L-arginyl-L-valyl-L-arginyl-L-arginine-7-AMC; Bachem) in 100 mM HEPES, pH 7.5 buffer containing 1 mM $CaCl_2$ and 0.5% Triton-X-100, at 37^0C, in 200 μl volume. Reactions were terminated by the addition of 100 μl 15 mM EDTA. 7-amino-4-methylcoumarin (AMC) liberated by cleavage was measured with a Perkin-Elmer LS-3 spectrofluorometer, 380 nm excitation, 460 nm emission. The blank sample consisted of substrate alone, while control milk values were negligible. One unit of furin activity was defined as the amount of enzyme liberating one picomole of AMC from the substrate per min. Specific activity was defined as units per mg of total protein. Assays were performed in triplicate. For kinetic analysis, duplicate milk samples diluted 1:200 were incubated for 5 min with boc-RVRR-AMC at concentrations ranging from 12.5 to 200 μM.

Whole milk from the HPC line 6.4 (2) was incubated in a 10:1 ratio with milk from HPC/PACE line C5.2, for 0-30 min at 37°C. Milk was then diluted with phosphate-buffered saline, 50 mM EDTA, pH 7.4 and processed as above. EDTA was used to chelate Ca^{++} and inhibit further furin activity, as well as to solubilize casein micelles and improve recovery of micelle-associated rHPC. Proteins were analyzed by SDS-PAGE and western blotting as described above.

Purification of rHPC. Whole milk, 0.9 ml, from the HPC line 6.4 was incubated with 0.1 ml milk from HPC/PACE line C5.2 and incubated for 3 hr, at 37°C. Milk was then diluted with 20 ml of 50 mM Tris, 0.15 M NaCl, 2 mM EDTA, 2 mM benzamidine, pH 7.2 and centrifuged at 30,000 g for 15 min at 4° C. The defatted milk was recentrifuged and filtered through a 0.45 μm Millex-HA (Millipore) membrane. The filtrate was loaded at a linear flow rate of 17 cm/hr onto a column of 8861 antibody immobilized on Sepharose CL-4B resin (Pharmacia) and equilibrated with 50 mM Tris, 0.15 M NaCl, 2 mM EDTA, 2 mM benzamidine, pH 7.2. The column was washed with the loading buffer, then with 5 mM ammonium acetate, pH 5.0. rHPC was eluted with 0.5 M ammonium acetate, pH 3.0, and immediately neutralized with 3 M Tris. Purified rHPC was subjected to automated Edman degradation and the N-terminal sequence determined.

Acknowledgements

We thank Drs. R.J. Kaufman and A. Rehemtulla for the human PACE/furin cDNA clone, Dr. D-W. Zhang for the generation of transgenic mice, Dr. M. Hans for assistance with purification, Dr. W.J.M. van de Ven for the generous gift of the MON 139, 148, 150, 152 antibodies, Dr.

L. Hennighausen for the anti-WAP antibody and Dr. C. Haudenschild for help with immunohistochemistry.

FIGURE 1. Immunohistochemical localization of furin in the mammary gland. Paraffin-embedded serial sections of mammary gland from mid-lactation, day 10, line C5.2 WAP/HPC transgenic mice were stained with hematoxylin/eosin (A) or probed with antibodies and developed by the indirect immunoperoxidase technique. Detection of mWAP with a rabbit anti-mouse WAP antibody (B), rHPC with a sheep anti-HPC antibody (C, E) and furin with the MON 148 antibody (D, F). Note that furin is present in the alveolar lumina and within epithelial cells (arrow), similar to rHPC and mWAP. The arrowhead marks the same alveolus in different sections. Magnification= 100X (A-D), 200X (E, F).

FIGURE 2. Furin is secreted into the milk of transgenic mice. Milk was collected from transgenic mice between days 7 and 15 of lactation. 20 μg of milk proteins were separated by SDS-PAGE in 8% gels under reducing conditions and western blot was probed with a 1:100 dilution of the MON 148 antibody. (A) Control mouse milk (CON), milk from HPC/PACE transgenic lines C1.2, C2.2, C4.1 and C5.2 (as marked). (B) Milk proteins from control and line C5.2 mice were western blotted with the monoclonal antibodies MON 139, 148, 150 and 152, as marked. Positions of molecular weight markers are shown on the left.

FIGURE 3. Secreted furin cleaves the rHPC precursor. (A) Milk from HPC/PACE or HPC mice, or a mixture of milk from HPC/PACE line C5.2 and HPC mice line 6.4 in a 1:10 ratio, was incubated for zero (lanes 1, 3, 5, 7, 9) or 30 mins (lanes 2, 4, 6, 8, 10) at 37^{0}C. Milk was then diluted in PBS-EDTA buffer, defatted and 20 μg of reduced proteins resolved on 8-16% gels. Western blot detection of rHPC was carried out using the 8861 monoclonal antibody (lanes

1-6) or the sheep polyclonal antibody (lanes 7-10). The dot indicates a mouse protein detected by the goat anti-mouse secondary antibody (lanes 1-6). (B) Purification of rHPC from the mixture of HPC/PACE and HPC mouse milk. Western blot detection of rHPC purified by immunoaffinity chromatography using the 8861 antibody and resolved by 8-16% SDS-PAGE was probed with the sheep anti-HPC antibody. rHPC purified from the milk of HPC/PACE mice, line C5.2 (lanes 1-2), from HPC transgenic mice (lanes 3-4) and from the mixture of HPC and HPC/PACE mouse milk, after incubation for 3 hr at 37°C (lanes 5-6), plasma-derived HPC (lane 7). SC: single chain, HC: heavy chain, LC: light chain. α, β, γ -HC represent HC glycoforms. (C) Amino terminal sequence of rHPC. Numbering corresponds to the first amino acid of the propeptide (-24), the light chain (+1) and the heavy chain (+158) of mature HPC.

FIGURE 4. Processing of rHPC and furin in the mammary gland. The WAP/HPC and WAP/PACE transgenes were coinjected and coexpressed in the mouse mammary gland. Pro-furin composed of the propeptide (P), serine protease (SP), middle (M), Cys-rich (CYS), transmembrane (TM) and cytoplasmic (C) domains, is activated by propeptide removal to the mature protein. Furin is localized to the Golgi, or undergoes C-terminal truncation and is secreted into milk. Pro-Protein C is processed intracellularly and in milk, by removal of the propeptide and dipeptide linking the light and heavy chains (LC, HC). The open arrows show known cleavage sites on the pro-proteins, while the dotted arrows indicate possible cleavage sites.

TABLE 1. Activity of furin in bigenic mouse milk.

Mouse Line	Activity per ml of milk (U/ml)*	Specific Activity (Units/mg total protein)	Michaelis constant, K_m (μM)
C 1.2	21,973	65.0 ± 2.1	32.26
C 2.2	27,470	64.5 ± 3.9	33.33
C 4.1	10,902	38.1 ± 1.3	32.15
C 5.2	40,515	128.2 ± 2.2	31.25

Activity was determined by cleavage of peptide substrate boc-RVRR-AMC, after 5 min incubation with defatted milk. The release of AMC was monitored at 460 nm. Results are representative of three separate assays.

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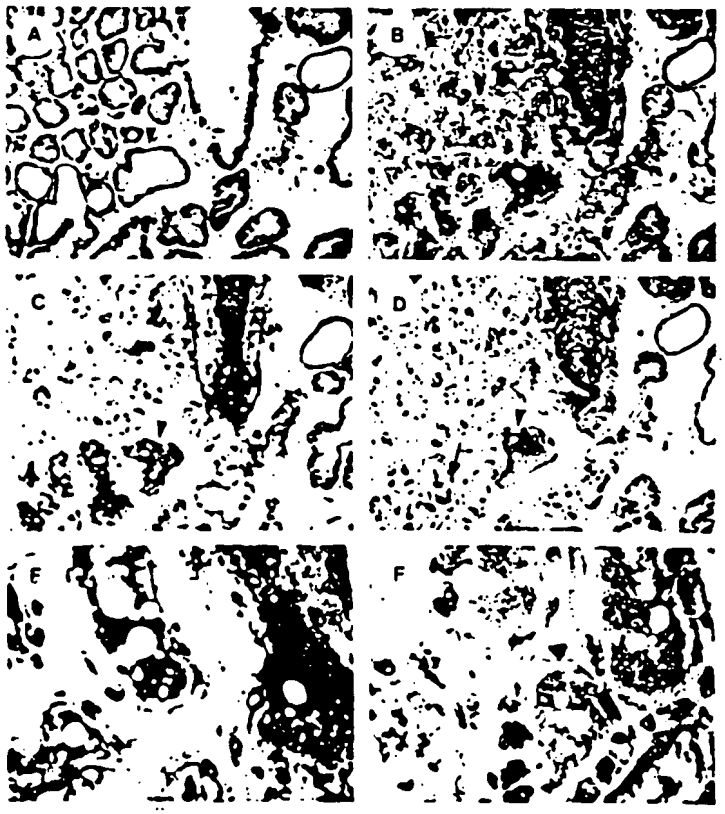

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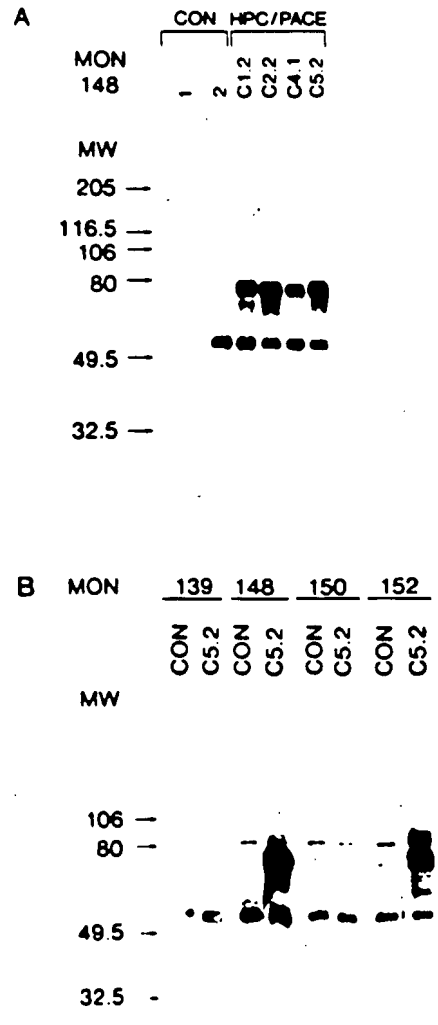
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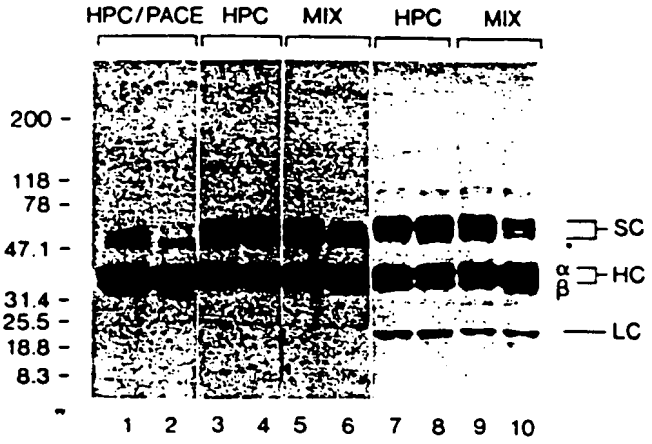

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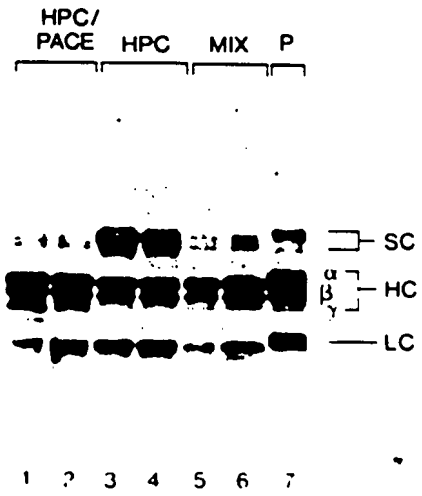
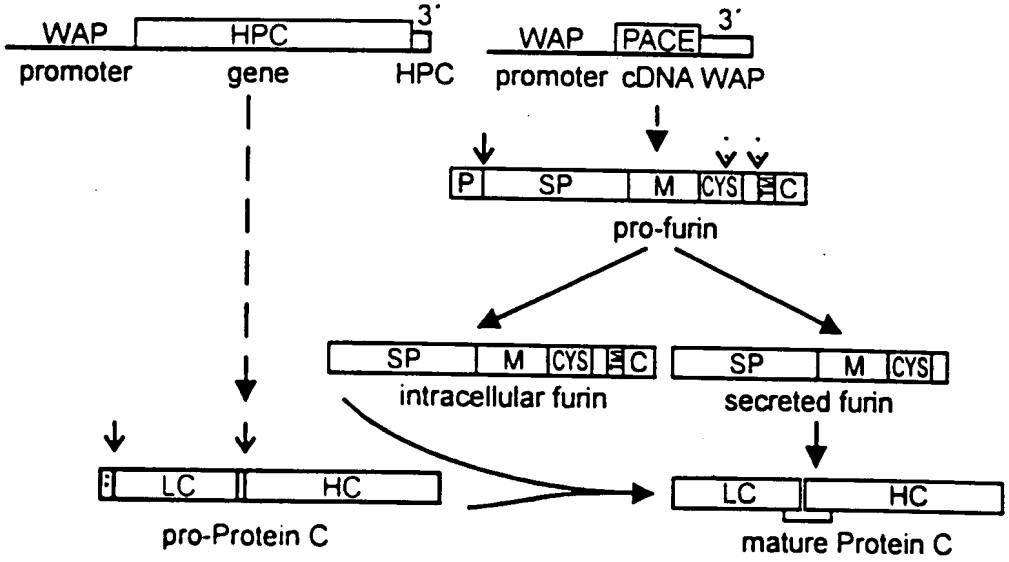


C

rHPC from the Milk of HPC Transgenic Mice		% Composition
Propeptide	$^{-24}$TPAPLDSVFSSS	20-30
Light chain	$^{+1}$ANSFLEELRHSSLEREC	70-80
Heavy chain	$^{+158}$DTEDQEDQVDPRLIDGK	100
rHPC from Mixture of Milk from HPC and HPC/PACE Mice		
Light chain	$^{+1}$ANSFLEELR	100
Heavy chain	$^{+158}$DTEDQEDQV	100

(4)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RESEARCH

HIGH LEVEL EXPRESSION OF ACTIVE HUMAN ALPHA-1-ANTITRYPSIN IN THE MILK OF TRANSGENIC SHEEP

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We describe the generation of five sheep transgenic for a fusion of the ovine β-lactoglobulin gene promotor to the human α_1-antitrypsin ($h\alpha_1AT$) genomic sequences. Four of these animals are female and one male. Analysis of the expression of $h\alpha_1AT$ in the milk of three of these females shows that all express the human protein at levels greater than 1 gram per liter. In one case initial levels exceeded 60 grams per liter and stabilized at approximately 35 grams per liter as lactation progressed. Human α_1AT purified from the milk of these animals appears to be fully N-glycosylated and has a biological activity indistinguishable from human plasma-derived material.

The prospect of producing large quantities of therapeutic proteins in the milk of transgenic livestock was publically raised some years ago[1,2]. As an alternative to cell culture systems, this production route is appealing because of the simplicity of access to the expressed protein, the high production capabilities of the mammary gland, the relatively low operating costs, and finally, the potentially unlimited expansion of the producer animals through established and emerging methods of animal husbandry. Like expression systems based on cultured mammalian cells, the mammary gland appears to be capable of performing the post-translational modifications vital to the activity or stability of certain pharmacologically active human proteins. In the last five years numerous publications have appeared attesting to the feasibility of this approach (for review, see ref. 3). Typically the gene of interest (either cDNA or genomic DNA) is fused to the regulatory sequences of the gene for a milk protein, and the fusion construct used to generate transgenic animals. In numerous cases the desired protein has been found in the milk, however the yields of protein have been extremely variable and usually much less than 1 gram per liter, although levels as high as 23 grams per liter in mice[4] and 2 grams per liter in pigs[5] have been obtained with foreign milk proteins, and 1–2 grams per liter of human urokinase, a plasminogen activator, has been produced in mouse milk[6].

Human α_1-antitrypsin ($h\alpha_1AT$) is a 394 amino acid glycoprotein which is normally present at 2 grams per liter in plasma[7]. The primary site of $h\alpha_1AT$ production in the body is the liver[7–9], and genetic deficiencies in circulating concentrations of $h\alpha_1AT$ are one of the most common lethal hereditary disorders to affect Caucasian males of European descent and sufferers are at risk of developing life-threatening emphysema. Replacement therapy using human plasma-derived α_1AT, has been sanctioned in the USA[7] where the large number (>20,000) of affected individuals and large amounts needed (~200 grams/patient/year[10]) make a strong case for an alternative, recombinant DNA-derived source, which is capable of yielding large quantities of $h\alpha_1AT$ and of performing the glycosylation events needed for plasma stability[10]. Recently Archibald et al.[11] reported yields of up to 7 grams per liter of biologically active $h\alpha_1AT$ in the milk of transgenic mice expressing a minigene containing the sheep betalactoglobulin (BLG) promotor fused to $h\alpha_1AT$ sequences, which comprise part of exon 1 and the remaining, downstream introns and exons, excluding intron 1. If similar high yields could be obtained in the milk of transgenic livestock, this could form the basis for a manufacturing process.

In this paper we demonstrate that the sheep mammary gland can offer a production route for large quantities of glycosylated, bioactive $h\alpha_1AT$. Using the $h\alpha_1AT$ minigene described above, we report the generation, lactation, and milk protein analysis of three founder transgenic animals. Two of the animals produce 1–5 grams per liter quantities of $h\alpha_1AT$ whilst the third produces ~35 grams per liter making $h\alpha_1AT$ the major protein in the milk. These levels have been sustained in all cases through 7 weeks of lactation and exceed by several orders of magnitude previously reported yields of foreign proteins in sheep milk and, in one case, the yield is substantially (>17 fold) higher than that reported for any foreign, non-milk protein in any transgenic system[3]. We believe these results confirm the feasibility of using mammals as bioreactors for the production of human therapeutic proteins.

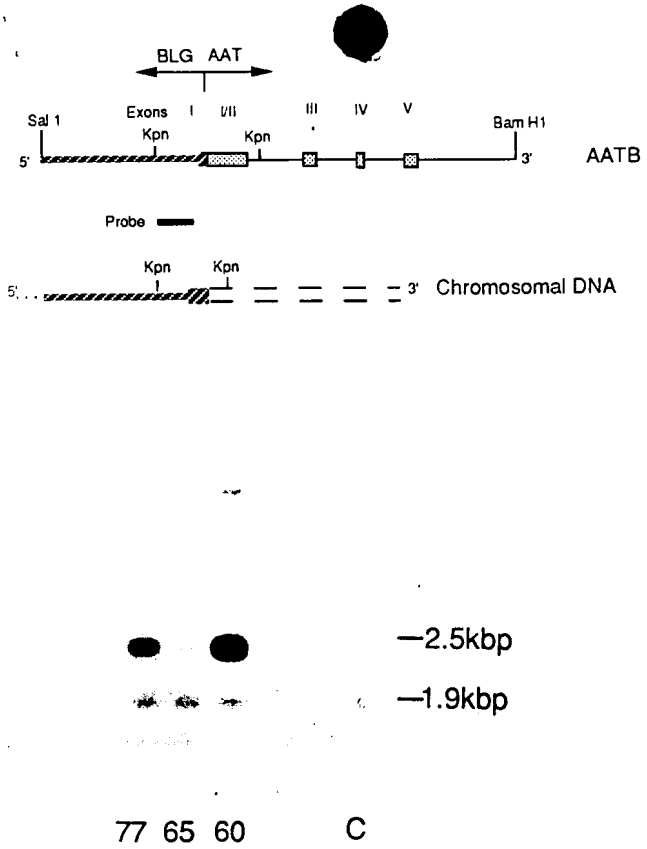

FIGURE 1 Southern blot analysis of transgenic DNA. The diagram shows the relevant region of the sheep chromosomal BLG locus as well as the intron/exon structure of the AATB construct used for injection (also see ref. 11); hatched regions correspond to BLG sequences and stippled regions and plain lines correspond to AAT sequences. DNA purified from the peripheral blood lymphocytes of transgenic sheep 60, 65 and 77 and a non-transgenic control animal (C) was digested with KpnI and analyzed as described in the Experimental Protocol. The membrane was hybridized with a radioactive probe homologous to 800 bp of the ovine BLG promoter (see diagram) and was generated by random priming (Stratagene). This reveals a 2.5 kbp internal band from intact transgenes and a 1.9 kbp band derived from endogenous BLG sequences (see diagram).

TABLE 1 Summary of the generation of transgenic sheep

Parameter	Value
No. eggs injected	549
No. eggs surviving	439
No. recipients	152
No. eggs per recipient	2.88
No. of pregnancies	73
No. births	113
No. screened	112*
No. transgenic	5
Percent of births transgenic	4.5
Percent of injected eggs transgenic	0.91

*One animal was stillborn and proved unsuitable for analysis.

RESULTS

Generation of transgenic sheep. Archibald et al.[11] recently described the production of bioactive hα_1AT in the milk of mice transgenic for a hybrid ovine BLG-hα_1AT gene, referred to as AATB. Of seven lines of animals expressing the AATB transgene at variable levels in the lactating mammary gland, four produced milk levels of greater than 0.5 grams per liter hα_1AT with one yielding a level in excess of 7 grams per liter. For this study, we made use of the same hybrid AATB construct.

Initially, we extended the observations of Archibald and colleagues by generating nine G_0 founder mice transgenic for the AATB fusion. Although levels of hα_1AT produced in the milk of these animals varies from line to line, all express the transgene at between 0.4 mg and 12.45 grams per liter. Moreover, the highest expressing animal has transmitted the transgene to her offspring and all G_1 females (three) exhibit a capacity similar to that of their mother to secrete hα_1AT with their milk (unpublished data).

These studies suggest that the AATB construct is efficient at directing the expression of hα_1AT to the lactating mammary gland with concomitant secretion of the human protein. To confirm that this is true not only in mice but also in sheep, we generated sheep transgenic for the AATB fusion gene. A total of 549 sheep eggs were microinjected with purified AATB DNA giving rise to 113 lambs (Table 1). One of these animals was stillborn and proved unsuitable for further analysis. Of the remaining 112, five proved to be positive for the AATB hybrid gene upon Southern blot analysis of genomic DNA samples. Four of these are female and one male.

These five animals developed normally and have shown no ill effects attributable to the presence of the transgene. To date, three of the females (nos. 60, 65 and 77) have produced offspring. Sheep 60 produced two female lambs, one of which is transgenic, sheep 65 produced one non-transgenic male and sheep 77 produced one non-transgenic female (data not shown). To assess the integrity of the incorporated transgenes in these three G_0 animals we performed Southern blot analyses of genomic DNA derived from peripheral blood lymphocytes. Cleavage of integrated copies with KpnI should release an internal fragment of 2.5 kbp (Fig. 1). This is revealed with a probe covering the first 800 bp of the BLG sequences present in AATB. The probe also reveals a 1.9 kbp band derived from endogenous ovine BLG sequences by hybridization to identical target sequences. Comparisons of band intensities with copy number controls (data not shown) and the endogenous BLG bands suggest that sheep 60 contains ~10 copies of the transgene, sheep 65 ~2 copies and sheep 77 ~5 copies. Analyses using other restriction enzymes and probes suggest that the multiple integrants contain intact copies of the transgene (data not presented). However, as previously found in transgenic sheep[12], the arrays are complex with both head to head and head to tail repeats. The elucidation of the exact structure of these arrays awaits further study.

Levels of human α_1AT in transgenic sheep milk. The offspring from animals 60, 65 and 77 were artificially reared and milk collected daily from their lactating mothers. Samples were pooled on a weekly basis and analyzed for the presence of hα_1AT. Initial determinations were performed with both a radial immunodifusion assay (RID) and ELISA. Neither of these techniques produce a cross-reaction with sheep α_1AT. A good correlation was observed between results obtained with these techniques and further determinations were performed using RID alone.

Levels of hα_1AT present in the milk of all three founder animals have exceeded 1 gram per liter (Table 2). There is no direct relationship between transgene copy number and levels of expression. It is notable, however, that yields do increase with increasing copy number. Sheep 60 produced 63 grams per liter hα_1AT in week one but has since stablized to yield ~35 grams per liter in

subsequent weeks. The human protein is consistently ~50% of the total protein in the milk of this animal. Sheep 65 produced 3.8 grams per liter in week one and has since stablized at around 1.5 grams per liter. Again this is a constant percentage of the total protein produced of about 3.5%. In contrast, Sheep 77 began secreting $h\alpha_1AT$ at 0.9 grams per liter and has since increased output attaining 3.5 grams per liter in week seven. This reflects an increase in the percentage of total protein that is $h\alpha_1AT$ from 1.4 to 10%. We have no explanation for this at present.

It should be noted that milk from week one contained colostrum and as such had higher concentrations of both $h\alpha_1AT$ and total protein. However, the total protein levels recorded for subsequent weeks has remained within observed limits for sheep milk despite being higher than expected for this breed (Blackface/Friesland). We are, therefore, not in a position to comment on whether endogenous protein production has been suppressed in these animals or whether total protein production has been increased.

Characterization of human α_1AT from transgenic sheep milk. Milk from founder animals 65, 77 and 60 was analyzed by SDS/PAGE (Fig. 2, lanes 2, 3 and 4 respectively). A novel band of apparent 54 kD molecular weight was observed in all three samples (indicated by arrow). This is the predicted molecular weight of native plasma derived $h\alpha_1AT$ (Fig. 2, lane 8). We confirmed this to be $h\alpha_1AT$ by western blotting (data not shown). Note that in the sample derived from sheep 60, the $h\alpha_1AT$ is the major protein in the milk.

Milk samples from all three sheep were defatted and $h\alpha_1AT$ was purified from the remaining material using anion exchange, dye affinity, hydrophobic interaction and gel filtration chromatography (manuscript in preparation). When analyzed by reducing (data not shown) and non-reducing SDS/PAGE (Fig. 2, lanes 5, 6 and 7) all three products migrate as a single band of about 54 kD similar to that observed for plasma derived $h\alpha_1AT$ (Fig. 2, lane 8). We estimate the purity of the three products to be >95% following silver staining, densitometry scanning and HPLC analysis (data not shown). Sheep milk naturally contains 1–2 μg per ml α_1AT. Our purified material could therefore contain a small percentage of sheep α_1AT that would not be revealed by our RID or ELISA assays, which are specific for the human protein. However, a comparison of the results obtained from these two techniques with total protein estimates indicated that our purified $h\alpha_1AT$ is at least 95% human protein. This is supported by amino terminal sequence data, which do not reveal any contamination with sheep α_1AT (manuscript in preparation).

Glycosylation of human α_1AT from transgenic sheep milk. Human α_1AT has three N-linked branched carbohydrate chains linked to asparagines 46, 83 and 247. Non-glycosylated recombinant $h\alpha_1AT$ is active but exhibits an accelerated *in vivo* plasma clearance[10], probably reflecting the absence of carbohydrate moieties. The apparent molecular weight of the material purified from transgenic sheep milk suggests that it is fully glycosylated, and to determine if this is so samples were cleaved with N-glycosidase F (Fig. 3). Lanes 1–4 contain uncleaved material and lanes 5–8 cleaved samples. In all cases, the $h\alpha_1AT$ purified from transgenic sheep milk behaves similarly to $h\alpha_1AT$ purified from human plasma (Fig. 3, lanes 1 and 8). Digestion of all samples results in a shift of electrophoretic mobility similar to that observed with human plasma derived α_1AT (Fig. 3, lanes 1 and 8). Furthermore, all of our purified material appears to be fully N-glycosylated (compare lanes 2–4 with lanes 6–8). Re-examination of the $h\alpha_1AT$ from complete milk (eg.

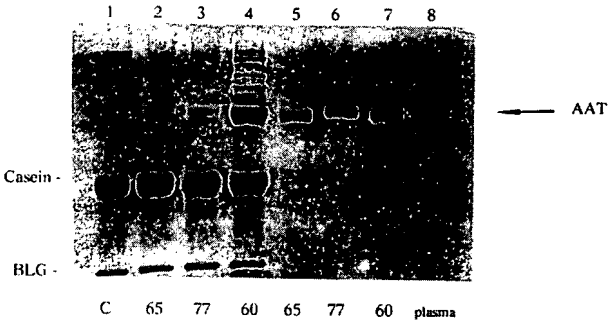

FIGURE 2 Non-reducing SDS-PAGE of transgenic sheep milk and purified $h\alpha_1AT$. Aliquots (0.1 μl) of whole milk from transgenic sheep 65, 77, 60 and a control (C) non-transgenic animal (lanes 1–4) or 1 μg of $h\alpha_1AT$ purified from the milk of transgenic sheep (lanes 5–7) were analyzed on a 12% non-reducing, SDS-PAGE gel as described in the Experimental Protocol. Lane 8 contains 1 μg $h\alpha_1AT$ purified from human plasma (Miles, Inc.). The running positions of casein and betalactoglobulin are indicated on the left of the figure. The position of plasma derived $h\alpha_1AT$ is indicated by the arrow on the right of the figure.

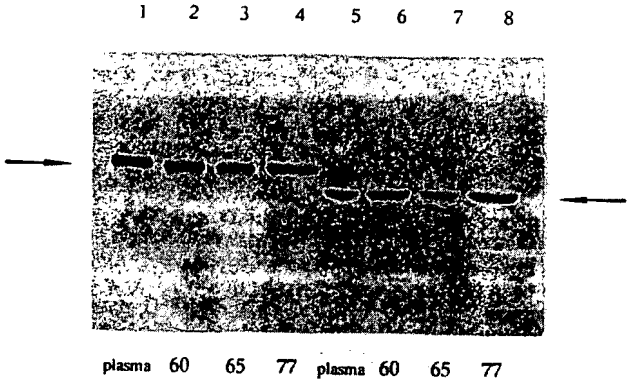

FIGURE 3 SDS-PAGE of glycosylated and deglycosylated $h\alpha_1AT$. $h\alpha_1AT$ (0.5 μg) samples purified from human plasma (Miles, Inc.) or from the milk of transgenic sheep 60, 65 and 77 were treated with (lanes 5–8) or without (lanes 1–4) N-glycosidase F as described in the Experimental Protocol. The arrow to the left of the figure indicates the position of glycosylated material (54 kD), the arrow to the right indicates the position of cleaved deglycosylated material (45 kD). MW markers are not shown.

TABLE 2 Analysis of human α_1AT in transgenic sheep milk.

Sheep	Week	Protein Content	h α_1AT Content
60	1	127.2	63.0
	4	66.8	31.7
	7	71.2	37.5
65	1	72.4	3.8
	4	44.1	1.3
	7	41.6	1.5
77	1	64.0	0.9
	4	44.4	2.2
	7	35.8	3.5

Animals were milked daily and the weekly produce was pooled prior to analysis. Figures from three representative weeks are presented in grams per liter of milk.

track 4, Fig. 2) shows that it corresponds in mobility to the glycosylated products shown in Figure 3 and therefore excludes the possibility that the purification was selective for glycosylated forms of α_1AT, indicating that all the hα_1AT is glycosylated. These results demonstrate the ability of the ovine mammary gland to N-glycosylate large quantities of secreted protein. We are currently determining the nature of these sugar moieties.

Bioactivity of hα_1AT purified from transgenic sheep milk. To analyze the activity of our purified hα_1AT we compared its ability to inhibit trypsin to that of two samples of plasma-derived hα_1AT using a colorimetric assay. A standard curve generated with one plasma-derived source was used to determine the activities of each of the other samples. In all cases, the hα_1AT purified from transgenic sheep milk shows similar activity to both plasma-derived products (Table 3).

DISCUSSION

We report in this paper the production in sheep milk of large amounts of a foreign protein, human α_1AT. We presume that this protein is made predominantly in the mammary gland for several reasons. First, analysis of the tissue-specificity of transcription from the AATB construct in mice indicates that the major site of transcription is the mammary gland, although in some animals a low level of expression from the salivary gland was noticed[11]. Second, if hα_1AT were synthesized outside the mammary gland, it would presumably gain access to the mammary gland via the blood. However circulating levels of hα_1AT are negligible compared to sheep α_1AT whereas this situation is reversed in the milk (data not shown). Direct analysis of RNA expression will be performed eventually, however at present we are concerned not to compromise the animals during their first lactation.

Concentrations up to 35 grams per liter of hα_1AT have been obtained. This level of hα_1AT production has now been sustained throughout the lactation period (twelve weeks); this situation contrasts with that recently reported for transgenic swine producing mouse whey acid protein where lactation itself was not sustained in 2 out of 3 lines as a result of transgene expression[5]. With milk yields per lactation ranging from 250–800 liters, according to sheep breed, the overall yield of hα_1AT per animal per lactation could exceed 10 kg. The hα_1AT recovered shows similar bioactivity to the human plasma-derived product. In addition, the hα_1AT produced by all three animals is fully N-glycosylated and we are presently investigating the exact sugar composition of the carbohydrate side chains. This demonstrates that in the mammary gland, the glycosylation apparatus has not been saturated by the requirement for hα_1AT glycosylation even though normally only a small proportion of the endogenous milk protein (<10%) is glycosylated and most of this represents O-linked glycosylation of κ-casein. Despite the commandeering of the animals' transcriptional and translational machinery for foreign protein production, all the transgenic sheep described here are perfectly normal and healthy. Although we have so far only been able to demonstrate transgene transmission in one of the three female sheep [the one transgenic male, has transmitted the transgene (data not shown)], the seven out of eight transgenic sheep previously generated by Clark and colleagues[12] have been found to transmit their transgenes in an unrearranged fashion (J. Clark, personal communication).

With few exceptions[13–15] it still remains the case that expression from the same transgene construct is highly variable between different lines. This has been attributed to various causes, including host genetic background, site(s) of chromosomal insertion, absence of certain transcriptional elements, etc[16]. Although there is no formal proof, we believe that the sheep BLG gene used to provide control elements for our transgene constructs has all the regulatory sequences necessary to confer high expression on a foreign gene fragment, since expression of the complete BLG gene in transgenic mice led to a range of yields but nearly all of them were high[4]. Although dramatically lower expression levels have been reported for fusion constructs between foreign genes and milk protein gene promotors including sheep BLG, this may be attributable in part to the absence of native introns in the foreign gene inserts[17]. Improvements in expression have been obtained when native, foreign or hybrid introns are added back[17–19]. When originally expressed in mice by Archibald et al.[11], the minigene used in our study gave hα_1AT yields of 80 milligrams −7.7 grams per liter with some animals not producing any detectable protein at all. Repeating this work we obtained a range of yields from 0.4 milligrams −12.45 grams per liter from nine different lines, a 30,000-fold range in variation. Although only 3 founder ewes have been analyzed in the study reported here (the fourth ewe is about to give birth), a 10-fold range of yields was obtained. While a comparison of the mice and sheep hα_1AT yields is questionable due to the small sample size, it is notable that the expression levels in sheep are on average higher and less variable. This may be a consequence of the homologous combination of an introduced sheep milk protein gene promotor operating in a sheep mammary gland environment.

In summary, we describe the production of high levels of a human therapeutic protein, α_1AT, in sheep milk. In one case the hα_1AT represents nearly 50% of total milk protein throughout the lactation period. These results indicate that it is possible to dramatically alter milk composition, opening up opportunities in the dairy industry to carry out a range of manipulations from over-expression of existing proteins to the introduction of novel milk proteins, which may allow improvements in milk formulations for both adult and infant consumption. In addition, this level of hα_1AT production exceeds those obtained in bacteria (15% total cell protein[20,21]), yeast (40% soluble protein[22]), and cultured mammalian cells (<1mg/10^6 cells/24h[23]), and provides a strong impetus to the further exploitation of transgenic sheep as bioreactors for the production of large amounts of pharmacologically active proteins.

TABLE 3 Bioactivity of hα_1AT purified from transgenic sheep milk.

Source of Purified hα_1AT	Percent Activity
Sheep 60 milk	95 ± 12
Sheep 65 milk	93 ± 22
Sheep 77 milk	86 ± 15
Human plasma α1AT (Miles, Inc.)	100
Human plasma α1AT (Sigma)	94 ± 5

Results are derived from 5 separate assays performed on two separate days and are normalized to the values obtained with the Miles, Inc. sample.

EXPERIMENTAL PROTOCOL

Generation of transgenic sheep. Transgenic sheep were generated essentially as described by Simons et al.[12] with the following differences: superovulation was induced with regimes of equine, porcine or ovine FSH; ovulation was synchronized in donor ewes (Scottish Blackface) using Receptal (Hoechst Animal Health); eggs were collected from donor ewes artificially inseminated with approximately 10^7 fresh, motile spermatozoa (Friesland) by intrauterine laparoscopy; eggs were collected by mid-ventral laparoptomy

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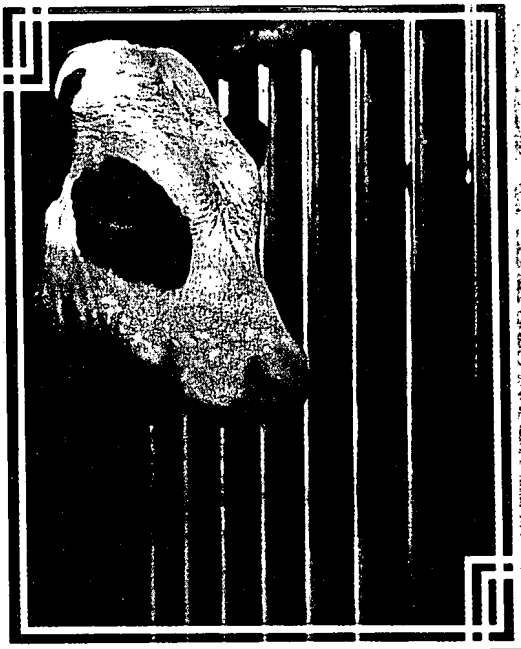

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TRANSGENIC CORNUCOPIA: PLUMS, CARNATIONS, AND MELONS

A NEW BACULOVIRUS INSECTICIDE

(5)

GENE TRANSFER INTO SHEEP

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Gene transfer into animals has considerable potential for livestock improvement. If this potential is to be realized, the ease of generation of transgenic livestock will be of major importance. We report here the production of six transgenic sheep by microinjection of DNA into early embryos (1.2% of embryos transferred). Three different gene constructs were injected and transgenic sheep were obtained with each. The transgenic animals have all incorporated the DNA without detectable rearrangement, and where multiple copies were integrated, they are present in arrays of tandem repeats. Transmission of transferred genes to progeny of three of the sheep has been demonstrated. Five founder transgenic sheep described carry genes designed to direct the production of human clotting factor IX or human α1-antitrypsin in milk. Transgenic animals carrying such genes may ultimately provide a new source of these and other therapeutic proteins.

Gene transfer into the mouse germline is now a routine procedure[1]. Typically, single-cell eggs are microinjected with DNA into one pronucleus, and are implanted into a foster mother. A proportion of the animals which develop from these eggs carry the injected DNA integrated into a chromosome. The foreign genes (transgenes) are often expressed correctly and can have profound phenotypic effects on transgenic mice, exemplified by the greatly increased size of transgenic mice with elevated levels of growth hormone in their serum[2-4]. Transgenic mice usually transmit the transgenes to their offspring, allowing large numbers of transgenic animals to be produced readily, by conventional breeding.

Gene transfer has considerable potential for the genetic improvement of farm animals. [5-9]. However, the generation of transgenic livestock not only presents a considerable technical challenge, but transferred genes must also confer economic advantage. While it is not yet clear which genes will have beneficial effects on conventional animal production traits (eg. reproductive performance, growth rate, carcase composition), we envisage the introduction of novel traits by gene transfer. One such trait may be the production of high-value proteins in the lactating mammary gland and their secretion into the milk for ease of collection[6-12]. The feasibility of such an approach is demonstrated by the secretion of foreign proteins into the milk of particular transgenic mice[11,12].

In view of the high cost and protracted time scale of generating transgenic cattle, we elected to pursue experiments in sheep[9,10]. While there have been few reports of transgenic livestock to date, two laboratories have generated transgenic pigs with efficiencies approaching those obtained in mice[8,13]. In contrast, two large experiments attempting gene transfer into sheep resulted in only one transgenic animal[6,7]. This sheep carried a single copy of the injected DNA, rearranged such that the gene could not be expressed[6]. These results have led to the speculation that sheep may be particularly refractory to gene transfer[7].

We report here gene transfer into sheep by microinjection of DNA into fertilized eggs, at a frequency similar to that obtained in mice. In each case the injected DNA was incorporated without detectable rearrangement, and three transgenic sheep have been shown to transmit the transgene to their progeny. Two gene constructs transferred into sheep (BLG-FIX and BLG-α1AT) are designed to direct the synthesis of human blood coagulation factor IX and human α1-antitrypsin in the lactating mammary gland and their secretion into milk.

RESULTS AND DISCUSSION

Production of transgenic sheep. Many factors influence the efficiency of production of transgenic mice by microinjection of DNA. Of particular importance are the site of injection of the DNA (pronuclei) and the form and concentration of the DNA[14] (linear, 1–2 μg/ml). We therefore chose to inject appropriate concentrations of linear DNA into the pronuclei of single-cell sheep eggs. Due to a lack of control of the precise timing of sheep early development, a number of 2- and 4-cell eggs were obtained, and these were also injected. Unlike mouse eggs, sheep eggs are semi-opaque and pronuclei and nuclei are not readily visible. Preliminary experiments showed that differential interference contrast (DIC) microscopy may be used to visualise pronuclei and nuclei in eggs that have been centrifuged, and that a proportion of such eggs are viable (data not shown). Subsequently, pronuclei were seen in untreated eggs (Fig. 1) and we have found that with careful DIC microscopy, pronuclei are visible in over 90% of eggs which have them (I. Wilmut and J. P. Simons, unpublished). A small number of eggs were injected after centrifugation, and centrifugation was discontinued when it was found that pronuclei could be visualized reliably without it. Details of the eggs injected and the animals obtained are summarized in Table 1.

Three DNA constructs (Fig. 2) were microinjected into sheep eggs. The first construct, pMK[15], contains the mouse metallothionein-1 (MT) promoter linked to the herpes simplex virus thymidine kinase (HSV-TK) gene in plasmid pBR322. This construct has been shown to function in transgenic mice[11,16]. The other constructs (BLG-FIX and BLG-α1AT) were based on the sheep gene encoding β-lactoglobulin (BLG), a major milk protein. The cloned sheep BLG gene[17] has been shown to be expressed specifically in the mammary glands of lactating

transgenic mice, directing the secretion of sheep BLG into mouse milk[11]. BLG-FIX and BLG-αIAT have cDNA sequences encoding human factor IX (FIX) or human α1-antitrypsin (αIAT) inserted into the 5' untranslated region of the BLG gene. These genes were designed to direct the synthesis of the human proteins in the sheep mammary gland and secretion into milk. pMK was linearized prior to microinjection, while the BLG-FIX and BLG-αIAT fusion genes were excised from the plasmid vectors because vector sequences have been shown to interfere with the expression of some transgenes[9,10].

A total of 511 microinjected eggs were implanted into recipient ewes and 119 fetuses were identified by ultrasound scanning at mid-gestation. DNA was obtained from 92 live-born lambs and from 17 dead fetuses (Table 1) and analysed by Southern blotting. Six transgenic lambs were identified (Fig. 3), and confirmed as such by blotting of DNA prepared from a second, independent blood sample. None of the fetuses analyzed was transgenic. One transgenic sheep carries pMK, four carry BLG-FIX and one BLG-αIAT. The estimated numbers of copies of DNA integrated in these animals ranges from 1 to 40 copies per cell (Table 2). All the transgenic sheep developed from eggs that had been injected at the one-cell stage; none of the 25 animals derived from the injection of 2- or 4-cell eggs was transgenic, suggesting that injection at these stages is less efficient for the generation of transgenic sheep. Brinster et al.[14] have found that injection into 2-cell eggs is less efficient for the generation of transgenic mice, in pigs the reverse may be true[41].

The frequency with which we obtained transgenic sheep is within the range of frequencies obtained by others with mice[14,15]. Two previous attempts to transfer DNA into sheep were less successful: 1) reimplantation of 1032 injected eggs generated 73 lambs and fetuses, one of which was transgenic[8], and 2) no transgenic fetuses were found among 71 analysed[7]. We obtained six transgenic sheep from 511 eggs that were injected and transferred (1.2% of eggs transferred). This improved frequency reflects both higher survival of injected eggs following reimplantation and a higher frequency of incorporation of the foreign DNA. It may be relevant that the protocol of Hammer et al.[8] included an extended period (>5h) of egg culture *in vitro*, whereas we reimplanted eggs as soon as possible after microinjection. It may also be significant that both of the earlier studies employed metallothionein-/growth hormone (MT/GH) fusion genes. Expression of MT/GH genes in transgenic mice is known to have deleterious pleiotropic effects on fertility[39] and it is possible that MT/GH expression may be detrimental to the ovine fetus. The fact that the single transgenic lamb obtained by Hammer et al.[8] carried a rearranged copy of the MT/GH gene is consistent with such an effect.

FIGURE 1 Visualization of sheep pronuclei. A single-cell sheep egg with both pronuclei clearly visible is shown immobilized on a holding pipette, for microinjection. The microinjection pipette is to the right. This is an exceptionally clear example: in most eggs the pronuclei are less distinct, and on occasion are invisible. In indistinct cases, pronuclei may be positively identified by visible distortion of the nuclear membrane on insertion of the microinjection pipette, and by the swelling which occurs on injection of DNA. Microscopy was performed with a Nikon Diaphot system.

Integrated transgenes are unrearranged. To assess the integrity of the incorporated transgenes, we performed Southern hybridization analyses on genomic DNA from each transgenic lamb. Transgenic animal 5LL229 was derived from an egg injected with BamHI-linearized pMK. Southern analysis of DNA from this animal after digestion with restriction endonucleases which cleave once or twice within pMK (Fig. 2), revealed hybridizing bands of sizes and intensities consistent with integration of a single unrearranged molecule of pMK (Fig. 3), which is intact at least from the left hand PvuII site to the EcoRI site near the right hand end (Fig. 2). In transgenic mice carrying pMK, the transgene is often, but not always expressed in the liver[13,16]. Despite the integrity of the pMK DNA integrated in 5LL229, no HSV-TK RNA was detected by Northern blotting of RNA from a liver biopsy (data not shown).

A similar analysis of transgene structure was performed on DNA from the four transgenic animals carrying the BLG-FIX fusion gene. The fusion gene could not be used as a hybridization probe because it contains repeated sheep DNA sequences within the BLG fragment. Instead, we probed separately with human FIX and sheep BLG cDNA sequences. The construct contains several EcoRI and BamHI sites (Fig. 2b). Cleavage with these enzymes gave the predicted hybridizing bands in all cases (Fig. 3b). In three animals, 6LL225, 6LL231 and 6LL240, the hybridization intensities revealed the presence of multiple copies of the injected DNA segment. Cleavage with Hin-

TABLE 1 Summary of eggs injected and animals obtained.

Construct	Stages of Injected Eggs					Animals Resulting	
	1-Cell	2-Cell (a)	2-Cell (b)	2-Cell (c)	4-Cell (d)	Lambs Born	Fetuses
pMK	29	6	14	22		26	9
pMK (e)	15			3		3	
BLG-FIX	252	27	17	4	7	52	5
BLG-αIAT	42	2			5	11	3
Total	402	35	31	31	12	92	17
Grand Total			511			109	

a: both cells injected, both cells survived.
b: both cells injected, one cell survived.
c: one cell injected.
d: one, two or three cells injected.
e: eggs centrifuged before injection.

dIII, which has a single recognition site in the construct (Fig. 2b), shows that the transgene is present in each of these animals both in head-to-tail and head-to-head repeats (Fig. 3b). The DNA incorporated in these sheep appears not to have undergone rearrangement. The hybridization intensity obtained with DNA from animal 6LL239 indicates the presence of one copy of the transgene per cell; the absence of HindIII bands corresponding to head-to-head or head-to-tail tandem arrays suggests that the estimated low copy number is not due to mosaicism.

Animal 6LL273 was produced by microinjection of the BLG-αIAT fusion construct. The insert was incompletely excised from the plasmid vector and the DNA injected contained approximately equal amounts of the BLG-αIAT (SalI-XbaI) insert of pSS1tgXS-αIAT and XbaI-linearized plasmid. Southern analysis of HindIII-cleaved 6LL273 genomic DNA revealed multiple copies of the intact linearized plasmid organized in a tandem head-to-tail array; bands that would have arisen from integrated multiple copies of the SalI-XbaI insert without vector were not observed (Fig. 3c). Hybridizing bands observed (Fig. 3c) using enzymes cleaving within the construct (EcoRI and BamHI) were as predicted from the restriction map (Fig. 2c). No rearrangement of the integrated construct was evident (Fig. 3c).

Transgenic sheep transmit the new genes to their progeny. To date, four of the transgenic sheep described above have produced offspring, which were analysed for the presence of the transgenes. Due to their immaturity, 6LL273 was not bred and 6LL239 did not sire any lambs.

Eighteen transgenic lambs were sired by 6LL225, from a total of thirty-eight offspring. Transgenic offspring of 6LL225 fall into three classes. The majority (15/38) are similar to 6LL225, with about 40 copies of the transgene per cell, two sheep have a low copy number, and one has an intermediate copy number (Fig. 4). Two possible explanations for this unexpected pattern of inheritance are that the integrated DNA may be unstable, or that 6LL225 carries transgenes integrated into more than one chromosomal site. While multiple sites of integration have commonly been found in transgenic mice[23], the lower copy number-transgenic offspring of 6LL225 were obtained at frequencies significantly less than 50% of scorable animals (1 out of 25, and 2 out of 22; $p<0.001$ and $p<0.001$). These frequencies could be explained by mosaicism of 6LL225 for the lower copy number-integration sites, or by integration into multiple sites on a single chromosome, with recombination occasionally occurring to separate sites of integration. Both male and female transgenic offspring of 6LL225 were obtained.

Transgenic ewe 6LL229 gave birth to one non-transgenic lamb, and ewes 6LL251 and 6LL240 each had one lamb (female and male, respectively), both of which are transgenic. Ewes 6LL251 and 6LL240 both carry the BLG-FIX transgene, designed to direct synthesis of human factor IX in the mammary gland during lactation. Preliminary analysis of milk from these animals indicates the presence of human factor IX (data not shown). The patterns of hybridization obtained with DNA from the offspring of 6LL251 and 6LL240 are indistinguishable from those of their mothers, suggesting that the integrated transgenes were inherited intact.

Some 30% of transgenic mice obtained from microinjected single-cell eggs have mosaic germlines and transmit the transgene to progeny at low frequency[23]. The remainder transmit the DNA at high frequency. To realize the full potential of gene transfer in livestock, it is essential that the integrated transgene is transmitted to the progeny of transgenic founder animals. Transgenic animals

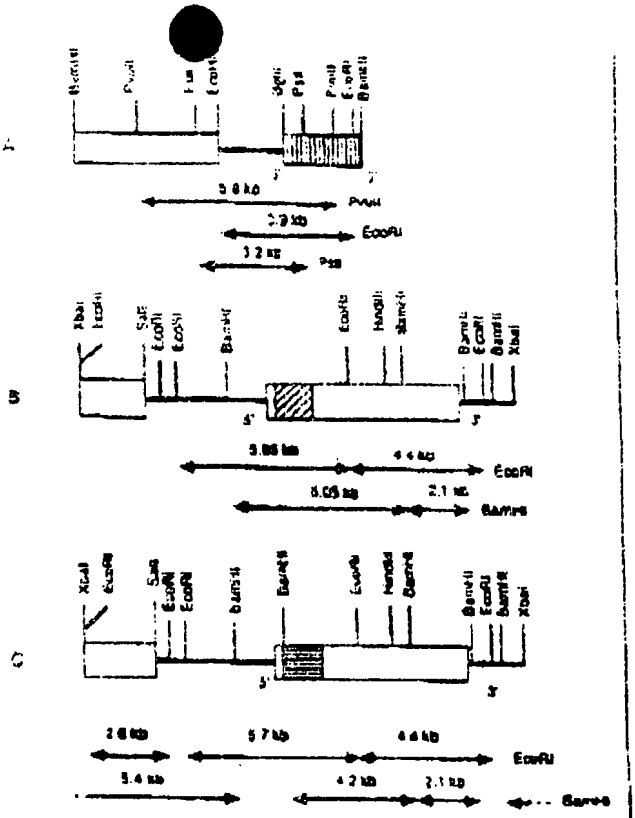

FIGURE 2 Restriction maps of microinjected constructs. A. Plasmid pMK[19]. Stippled box: pBR322 vector; solid line: mouse MT-1 promoter segment; vertical shaded box: HSV TK gene. Fragments relevant to the analysis of the transgenic sheep are indicated below the map. pMK was injected after linearization with BamHI. B. BLG-FIX. BLG-FIX, the insert of pSS1tgXS-FIX, is a 10.5 kb XbaI-SalI fragment from BLG genomic clone SS1[11,17], comprising the 4.9 kb transcription unit and flanking sequences, with a cDNA encoding human Factor IX (derived from p5'G5'cVI[18], 1.58 kb NheI-HindIII fragment) inserted into a PvuII site in the 5' untranslated region of the BLG gene. The predicted transcript of this fusion gene is a bicistronic mRNA (5'-FIX-BLG-3'). Stippled box: vector pPoly1[24]; open boxes: BLG transcription unit; solid lines: BLG flanking sequences; diagonal shaded box: FIX cDNA sequences. Beneath the map, the origins of the BamHI and EcoRI fragments detected (Fig. 3b) with the FIX and BLG cDNA probes are shown. The fragment injected was the Xba-SalI insert. C. BLG-αIAT. BLG-αIAT, the insert of pSS1tgXS-αIAT is analogous to BLG-FIX except that in place of the FIX cDNA a human αIAT cDNA was inserted (p5α1ppg (a gift of R. Cortese), 1.9 kb TaqI-BstNI fragment). Horizontal shaded box: αIAT cDNA sequences. The fragment was injected as a mixture of XbaI-SalI insert and XbaI-linearized pSS1tgXS-αIAT. Beneath the map, the origins of the BamHI and EcoRI fragments detected (Fig. 3c) by probing with αIAT and BLG cDNA clones are shown.

obtained by injecting 2- or 4-cell eggs are most likely to be mosaic. This is undesirable, especially in species which have small litters such as sheep and cows. Together with our failure to obtain transgenic sheep by injection of 2- and 4-cell eggs, this argues for injection only at the pronuclear stage.

In conclusion, we have demonstrated that gene transfer into the germ-lines of sheep may be reliably accomplished by microinjection of DNA into pronuclei of fertilized eggs. Transgenes are integrated without rearrangements, and the efficiency with which transgenic sheep may be obtained is sufficiently high to suggest that gene transfer into this species is practicable.

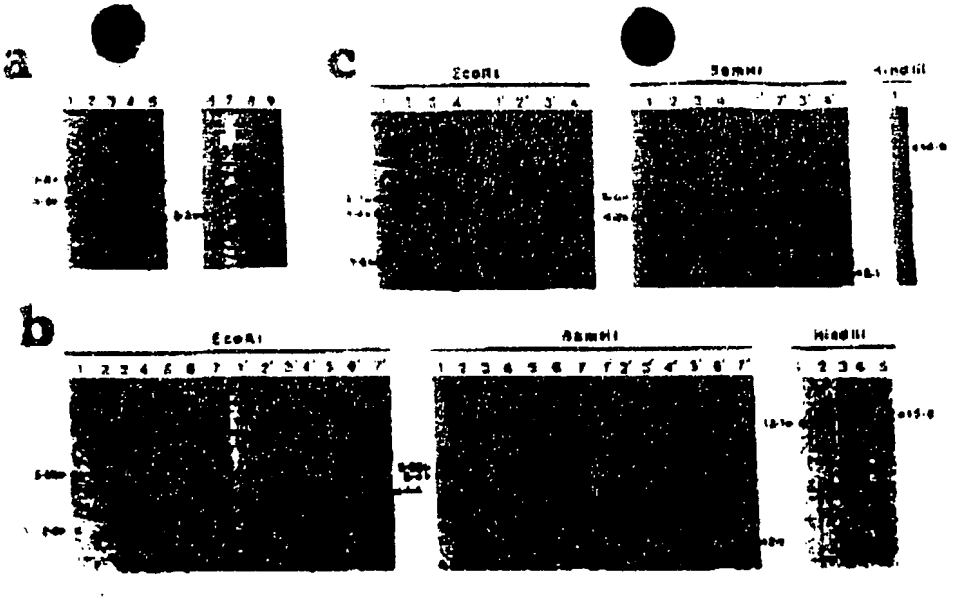

FIGURE 3 Southern analysis of transgenic sheep DNA. A. pMK. lane 1, control sheep DNA; lanes 2, 4, 6, and 8, control DNA plus 1 copy equivalent of pMK; lanes 3, 5, 7 and 9, DNA from 5LL229. The DNA was digested with EcoRI (lanes 1, 4, and 5), PvuII (lanes 2 and 3), PstI (lanes 6 and 7) and BglII (lanes 8 and 9). The hybridization probe was plasmid pTK1[39]. PvuII, EcoRI and PstI each cleave pMK twice and digests of 5LL229 DNA yielded the predicted hybridizing internal fragments. PvuII, 5.8 kb; EcoRI, 3.9 kb; PstI, 3.2 kb (see Fig. 2a). The BglII digest gave two hybridizing fragments neither of which coincides with linear pMK. These are presumed to originate from fragments extending from the single internal BglII site in pMK to flanking chromosomal BglII sites. B. BLG-FIX. DNA samples were cleaved as indicated. EcoRI- and BamHI-cleaved DNA: lanes 1, control sheep DNA; lanes 2 and 3, control DNA plus 1 and 5 copy-equivalent of pSSIIgXS-FIX; lanes 4–7: DNA from transgenic sheep 6LL225, 6LL231, 6LL239 and 6LL240. The filters were hybridized with the human FIX cDNA clone p5'G3'cVI (lanes 1–7) and subsequently with the BLG cDNA clone pβ-Lg931[36] after stripping (lanes 1'–7'). Each transgenic sheep yields FIX-hybridizing bands of 5.95 kb (EcoRI) and 6.05 kb (BamHI) identical in size with those derived from BLG-FIX, showing that the 5' ends of the integrated BLG-FIX transgenes are intact. Significant hybridization with the sheep FIX gene was not observed. Hybridization with the BLG probe revealed the predicted 4.4 kb EcoRI and 2.1 kb BamHI bands. Although the endogenous BLG genes contribute to the hybridization in these bands, the increased intensity in samples from 6LL225, 6LL231 and 6LL240 indicates that these bands derive primarily from the foreign DNA, confirming the integrity of the 3' ends of the transgenes. HindIII cleaved DNA: lane 1, 12.1 kb SalI-XbaI BLG-FIX fragment; lanes 2–6 DNA from 6LL225, 6LL231, 6LL239 and 6LL240. The probe was the FIX cDNA clone. The hybridizing 12.1 kb HindIII fragment (6LL225, 6LL231 and 6LL240), identical in size with the injected fragment, indicates a head-to-tail arrangement; the 15.6 kb fragment common to the same sheep indicates that head-to-head repeats are also present. C. BLG-α1AT. DNA samples were digested as indicated. EcoRI- and BamHI-cleaved DNA: lanes 1, control sheep DNA; lanes 2 and 3, control DNA plus 5 and 1 copy equivalents of pSSIIgXS-α1AT; lanes 4, 6LL273 DNA. The filters were hybridized with human α1AT cDNA clone pδα1ppg (lanes 1–4) and, after stripping, with BLG cDNA clone pβ-Lg931 (lanes 1'–4'). 6LL273 DNA digested with EcoRI or BamHI gives α1AT hybridizing bands of 5.7 and 2.6 kb and 5.4 and 4.2 kb respectively, as obtained on cleavage of pSSIIgXS-α1AT, showing the integrity of the 5' end of the fusion gene. As before, the intensity of hybridization of the 4.4 kb EcoRI and 2.1 kb BamHI fragments with pβ-Lg931 indicates their origin is primarily from the unrearranged 3' end of the transgene. HindIII cleaved 6LL273 DNA (lane 1) yields a 14.0 kb fragment hybridizing with pδα1ppg, indicating a head-to-tail arrangement of linear pSSIIgXS-α1AT DNA.

EXPERIMENTAL PROTOCOL

DNA for microinjection. pMK[39] was prepared for microinjection by BamHI digestion, phenol-chloroform extraction and ethanol precipitation. BLG-FIX and BLG-α1AT fusion genes were released from the plasmid vectors by digestion with SalI and XbaI, electrophoresed on 0.5% agarose gels and electroeluted onto dialysis membranes. Isolated fragments were purified on elutip D columns (Schleicher and Schüll), redissolved at about 100 μg/ml in 0.2 μm-filtered 10mM Tris.HCl, 1 mM EDTA (pH 7.5), and stored frozen. For microinjection, the DNA was diluted to 2 μg/ml (pMK) or 1.5 μg/ml (BLG-FIX and BLG-α1AT) in 0.2-μm-filtered double distilled water. Micropipette tips used to handle the DNA were rinsed thoroughly with filtered double distilled water to remove particles that could block microinjection pipettes. The DNA solution was centrifuged at 10,000 g for 30 min.; an aliquot withdrawn from below the surface was used to fill injection pipettes.

Collection of eggs. Animals used as egg donors were ewes of proven fertility of a variety of breeds: Welsh Mountain, Scottish Blackface, Greyface (Scottish Blackface × Border Leicester), and Cheviot. Rams of proven fertility were of several breeds: Finnish Landrace × Dorset Horn, Scottish Blackface and East Friesland. Donor ewes were treated with progestagen sponges (Veramix, Upjohn Ltd., Crawley, UK.) for 12–16 days to synchronize the time of estrus. Superovulation was induced by subcutaneous injection of equine FSH[39] in 2 equal doses of 1.75 or 2.15 mg, 30h before and at the time of sponge withdrawal (noon). Ewes were tested for the onset of estrus at 0800h, 1200h, 1600h and 2000h. At estrus, each ewe was served by at least two rams. Eggs were recovered[39] by flushing oviducts with prewarmed ovum culture medium (Flow Labs., Irvine, UK) during mid-ventral laparotomy.

Microinjection of DNA and reimplantation of eggs. Microinjection was performed using essentially standard techniques[40]. Eggs were manipulated in cover-slip chambers in ovum culture medium supplemented with 20% fetal calf serum. The ends of the chambers were covered with Dow Corning 200/50cs fluid.

TABLE 2 Summary of transgenic sheep.

Lamb No.	Sex	Construct	Copy no. (Approx.)
5LL229	F	pMK	1
6LL225	M	BLG-FIX	40
6LL231	F	BLG-FIX	10
6LL239	M	BLG-FIX	1
6LL240	F	BLG-FIX	10
6LL273	F	BLG-α1AT	4

All of the eggs were injected at the pronucleus stage and none were centrifuged. Copy numbers were determined by quantitative scanning densitometry of Southern blot autoradiographs.

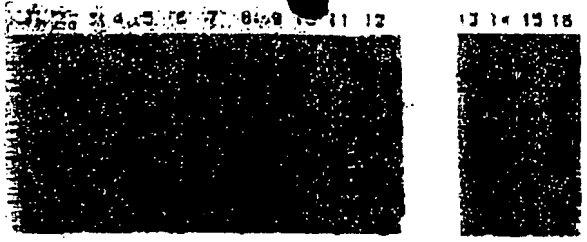

FIGURE 4 Germline transmission of incorporated DNA. Southern blot analysis of transgenes in offspring of BLG-FIX transgenic sheep 6LL225. The samples in lanes 1 to 12 were digested with EcoRI and probed with the FIX cDNA clone p5'G3'cVI. Lanes 1 to 11 contain DNA from representative progeny of transgenic sheep 6LL225, lane 12 contains DNA from 6LL225. Lanes 2, 8, 10 and 11 are representative of 20 of the 38 offspring: the transgene is not present. Lanes 1, 3, 4 and 6 are representative of 15 progeny, showing about 40 copies per cell of BLG-FIX. Lanes 5 and 9 show DNA from the two animals which have a low number of copies of the FIX sequences, and lane 7 shows DNA from the one son of 6LL225 which carries an intermediate number of copies of the transgene. In each transgenic sheep, the diagnostic 5.95 kb fragment was present. Lanes 13 to 16 contain DNA after digestion with HindIII and probing with p5'G3'cVI. The samples are from animals representative of the four classes of offspring, the same animals as shown in lanes 5 to 8. In the offspring which carry about 40 copies per cell and those which have a low copy number, the fragments diagnostic of head-to-tail (12.1kb) and head-to-head (15.6kb) repeats are both present, as found with the parent animal. Lane 15 shows DNA from the one offspring which carries about 5 copies per cell of BLG-FIX; the fragment diagnostic of head-to-head repeats is absent. Lane 16 contains DNA from a non-transgenic animal.

Microinjection pipettes were very fine (<0.5 μm) and tapered at a small angle (≤5 degrees), to minimise lethal damage to the eggs. They were pulled from thin wall borosilicate glass (1.0 mm O.D., Kwik-fil, Clark Electromedical Instruments, Pangbourne, UK). Single-cell eggs had DNA injected into one pronucleus, 2-cell eggs into both nuclei (season 1985/86) or into one nucleus (1984/85, or when only one was visualized, 1985/86). Injection of 4-cell eggs was into one, two or three nuclei (1985/86). When 2- or 4-cell eggs were injected more than once, injections were performed without repositioning the eggs on the holding pipette to avoid confusion over which cells remained to be injected. Successful injection was indicated by marked swelling of pronuclei or nuclei. Injected eggs were incubated for at least 30 min. before transfer, to allow damage to become apparent. Some eggs were centrifuged prior to injection at 10,000 g for 10 min. or for 5 min. after 30 min. incubation in medium containing 0.1 μg/ml colcemid. Egg recipients (Welsh Mountain ewes) were treated with progestagen sponges (Veramix, Upjohn, Crawley, UK.) for 12–16 days to synchronise their estrus cycles with those of the egg donors. Injected eggs were implanted as deeply as possible into the oviducts of recipients which had ovulated. 1–3 eggs were transferred per recipient (1985/86 or 1–4, 1984/85 or when the recipients were one day out of synchrony 1985/86). The embryos were distributed between the oviducts.

DNA Analysis. Samples of DNA (10 μg) from peripheral blood lymphocytes were analysed by restriction enzyme digestion, gel electrophoresis (1% agarose), Southern transfer to nylon membranes (Hybond N, Amersham) and hybridization using standard procedures.

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